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RESEARCH ARTICLE

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Sonic hedgehog expression in zebrafish forebrain identifies the teleostean pallidal signaling center and shows preglomerular complex and posterior tubercular dopamine cells to arise from *shh* cells

Mario F. Wullimann | Kosisochukwu E. Umeasalugo

Department Biology II, Division of Neurobiology, Ludwig-Maximilians-Universität München (LMU Munich), Munich, Germany

Correspondence
Dr Mario F. Wullimann, Department Biology II, Division of Neurobiology, Ludwig-Maximilians-Universität München, Grosshadernerstr. 2, 82105 Martinsried-Planegg, Germany.
Email: wullimann@bio.lmu.de

Abstract

Ventralization, a major patterning process in the developing vertebrate neural tube (central nervous system, CNS), depends on Sonic hedgehog (SHH) as a main signaling morphogen. We studied the CNS of late larval and young adult zebrafish in a transgenic *shh*-GFP line revealing increased neuroanatomical detail due to the progressed differentiation state compared to earlier stages. Some major findings emerge from the present study. (a) *shh* –GFP is still expressed along the adult zebrafish CNS neuraxis in most locations seen in larvae. (b) We newly identify a ventroposterior *shh* pallidal domain representing the basal telencephalic signaling center important for basal ganglia development known in other vertebrates (i.e., the anterior entopeduncular area—basal medial ganglionic eminence of mammals). (c) We further show late-emerging *shh*-GFP positive radial glia cells in the medial zone of the dorsal telencephalon (i.e., the teleostan pallial amygdala). (d) Immunostains for tyrosine hydroxylase demonstrate that there is selective colocalization in adult dopamine cells with *shh*-GFP in the posterior tuberculum, including in projection cells to striatum, which represents a striking parallel to amniote mesodiencephalic dopamine cell origin from *shh* expressing floor plate cells. (e) There is no colocalization of *shh* and *islet1* as shown by respective *shh*-GFP and *islet1*-GFP lines. (f) The only radially far migrated *shh*-GFP cells are located in the preglomerular area. (g) There are no adult cerebellar and tectal *shh*-GFP cells confirming their exclusive role during early development as previously reported by our laboratory.

KEYWORDS

floor plate, islet1, longitudinal gene expression, mesodiencephalic dopamine cells, pallium, posterior tuberculum, preglomerular complex, RRID: AB 2201528, RRID: AB 2340817, RRID: AB_10000240, RRID: AB_2340364, striatum, telencephalon, tyrosine hydroxylase, ventralization

1 | INTRODUCTION

Ventralization versus dorsalization represent major interdigitating patterning processes in the developing vertebrate neural tube (central nervous system, CNS). Hereby, morphogens are issued dorsally (roof plate) and ventrally (in two steps, first from notochord and prechordal mesoderm, later from floor plate and ventral forebrain cells; see section 4) in order to interact on neural cells of alar and basal plates with the result of a graded neurocellular fate along the dorsoventral CNS axis (Balaskas et al., 2012; Briscoe, 2009; Briscoe & Novitch, 2008; Briscoe & Small, 2015; Dessaud, McMahon, & Briscoe, 2008; Grossmann, Giraudin, Britz, Zhang, & Goulding, 2010; Martí & Bovolenta, 2002). Sonic hedgehog (SHH) is the main ventral signaling molecule (morphogen). We already summarized the hedgehog signaling pathway and the roles of three different zebrafish hedgehog genes, and also analyzed the larval expression of the sonic hedgehog gene (*shh)* using an established transgenic *shh*-GFP line (see section 2) in two recent previous papers (Baeuml, Biechl, & Wullimann, 2019; Biechl, Dorigo, Köster, Grothe, & Wullimann, 2016). These and other zebrafish brain expression studies (Ekker et al., 1995; Ertzer et al., 2007; Hagemann & Scholpp, 2012; Hauptmann & Gerster, 2000; Hauptmann, Söll, & Gerster, 2002; Holzschuh, Hauptmann, & Driever, 2003; Krauss, Concordet, & Ingham, 1993; Strähle, Blader, & Ingham, 1996; Wilson & Rubenstein, 2000) remain in line with general knowledge in vertebrates (see section 4).

However, there are two unresolved problems regarding *sonic hedgehog* expression in the zebrafish brain. One is the lack of a telencephalic (subpallial) *shh* expression domain comparable to what is described in amniotes as the anterior entopeduncular area (AEP) and medial ganglionic eminence (MGE; see section 4). These amniote telencephalic *shh* domains are crucial for correct ventral telencephalic gene expression (e.g., *Dlx*, *Ascl1*, *Nkx2.1*, *Islet1*, *Lhx6/7*) and, thus, for correct basal ganglia development as well as for repressing dorsal (i.e., pallial) gene expression ventrally (see section 4). A second issue is the developmental role of SHH in the generation of basal diencephalic dopamine cells. In mammals, midbrain substantia nigra/ventral tegmental dopamine cells are known to derive from sonic hedgehog expressing floor plate cells (Joksimovic et al., 2009; Blaess et al., 2011; Hayes, Zhang, Albert, Zervas, & Ahn, 2011; see section 4). Since teleosts lack midbrain dopamine cells and only possess basal diencephalic dopamine cells (posterior tuberculum)—which also contribute to the SN/VT in mammals (see section 4)—that nevertheless project to the fish basal ganglia (review Wullimann, 2014), we wanted to verify whether these zebrafish diencephalic ascending dopamine cells are produced by *shh* expressing cells.

Thus, we looked at early adult (3-month-old) transgenic *shh*-GFP zebrafish and described in neuroanatomical detail all GFP-positive CNS structures. Because the advanced differentiation state of the adult brain allows for a far more detailed identification of *shh*-GFP structures compared to larvae, we anticipated that the data will shed light onto both the recognition of a true pallidal *shh* domain as well as on the origin of dopaminergic posterior tubercular projection neurons to the teleostean striatum (see section 4.4.) from *shh* expressing cells from which part of the preglomerular area is also revealed to derive.

2 | MATERIALS AND METHODS

2.1 | Transgenic zebrafish strains

The transgenic line Tg(2.4*shha-ABC-GFP*)sb15 was originally published as Tg(*2.2shh*:*gfpABC#15*) by Shkumatava, Fischer, Müller, Strähle, and Neumann (2004). The injected construct includes the *sonic hedgehog* promoter (*Sal*I/*Xho*I fragment) upstream of *gfp* as well as intronic sequences for required enhancer regions (Müller et al., 1999). The line will be referred to in the following as *shh*-GFP line. Our lab has used it previously to study the larval expression of *shh*-GFP (Biechl et al., 2016). Here, we raised zebrafish *shh*-GFP specimens into larval stages and up to 3 months (early adults). Fish were maintained according to standard protocols (Westerfield, 2007).

The *shh*-GFP transgenic zebrafish line used here has previously been characterized to faithfully represent *shh* expression (Biechl et al., 2016; Shkumatava et al., 2004) in zebrafish retina and brain/spinal cord and the transgenic expression patterns are furthermore well in line with known *shh* expression patterns in other vertebrate species (reviewed in Biechl et al., 2016 and Baeuml et al., 2019).

The transgenic *islet1*-GFP line Tg(*isl1*:*GFP*) was originally generated by Higashijima, Hotta, and Okamoto (2000) by fusing *gfp* sequences with *islet-1* promoter sequences (ICP) to produce the core plasmid and adding enhancer elements (CM) for the construct that proved sufficient for specific neural expression. This line will be referred to here as *islet1*-GFP line. Details for the generation of these specimens, as well as the origin of brain sections depicted in this contribution, are given in a previous paper reporting on *islet1*-GFP expression (Baeuml et al., 2019).

All procedures involving live zebrafish were carried out according to EU guidelines and German legislation (EU Directive 2010_63, license number AZ 325.1.53/56.1-TU-BS). Transgenic animals used in this study were killed with an overdose of tricaine methanesulfonate (MS-222) and fixed in paraformaldehyde (4% PFA in Sörensen's phosphate buffer, PB) at 4°C overnight. The raising and fixation of transgenic animals were performed in Prof. Reinhard Köster's lab (Technical University Braunschweig, Germany) and kindly subsequently provided to us. Therefore, the present study only involved fixed animal tissue and needed no further approval.

2.2 | Cutting procedure

Following cryoprotection in sucrose solution (30% sucrose solution at 4°C overnight), the brains (heads) of adult *shh*-GFP zebrafish were embedded in TissueTek (tissue freezing medium, A. Hartenstein GmbH) and cryosectioned (Leica, CM 3050S) at 30 μm in the transverse or sagittal plane before thaw mounted onto Superfrost Plus glass slides (Thermo) and coverslipped after immunoprocedures. In total, 18 zebrafish specimens were used in this study, that is, one specimen each of 3–8 days postfertilization (dpf) larvae, four 13 dpf larvae, and eight 3-month-old specimens. Additionally, various 2, 3, 4, and 5 dpf *shh*-GFP specimens were available from a previous study (Biechl et al., 2016).

2.3 | Immunohistochemical processing

Immunohistochemical incubations were done in a humid chamber. After washing off TissueTek in cryosections with phosphate-buffered saline (PBS) the sections were blocked with blocking buffer (2% normal goat serum, 2% bovine serum albumin, 0.2% Tween20, 0.2% TritonX-100 in PBS) for 1 hr at RT before exposure to a primary antibody against GFP diluted in blocking buffer at 4°C for 1–3 days (dilutions see Table 1). After washing in PBT (PBS + 0.1% Tween 20), the sections were incubated with the secondary antibody (see Table 1) diluted in blocking buffer solution overnight at 4°C. Subsequently, a second primary antibody against tyrosine hydroxylase (TH; see Table 1) was applied after intermittent washing in PBT and blocking (see above for details), followed by the application of the appropriate secondary antibody (see Table 1) diluted in blocking buffer overnight, after intermittent washing in PBT and blocking (see above). Finally, sections were washed in PBT and counterstained with DAPI (4′-6-diamidino-2-phenylindole; Carl Roth, 1:1000) and washed in PBS. Slides were then mounted with Vectashield (Vectorlabs) or ProLong Diamond (Invitrogen/Thermo Fisher) and coverslipped. Previously, various controls and Western blot analysis for the antibody against TH have been performed (Yamamoto, Ruuskanen, Wullimann, & Vernier, 2010, 2011).

Furthermore, there were no neuroanatomical differences between the intrinsic GFP signal with the one enhanced through the use of the anti-GFP antibody.

2.4 | Photography

Cryostat sections of adult zebrafish heads were photographed using a light/fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Inc.) with a Nikon Digital Sight DSU1 Photomicrographic Camera (Nikon Instruments Inc.) and NIS-Elements F4.60.00 software. The microscope was equipped with Nikon Plan UW 0.06 (2×), Plan Fluor DIC L/N1, ∞/0.17, WD 16.0 (10×/0.30)) and Plan Fluor DIC M/N2, ∞/0.17, WD 2.1 (20×/0.50) objectives.

All images were eventually slightly adapted for brightness and contrast with Corel PHOTO-PAINT 9.0 and mounted into figures with Corel DRAW 9.0 (Corel Corporation, Ottawa, Canada).

2.5 | Analysis of data

Most sections were photographed in three appropriate fluorescent spectral channels for the presence of the nuclear stain DAPI, *shh*-GFP or *islet1*-GFP, and TH. In cases where the GFP and TH label was in the same area, the ImageJ tool of synchronizing all windows was used to analyze cellular colocalization of *shh*-GFP with TH on a neuroanatomical background yielded by the DAPI pictures. Since the three microphotographs were identical in each case except for the fluorescence visualized, we could assign in detail to a cell nucleus seen in DAPI stain the associated cytoplasmic green GFP and red transmitter-related enzyme stain on a cell to cell basis.

3 | RESULTS

The *shh*-GFP is generally still expressed in early adult zebrafish brains in most locations along the neuraxis as seen in larval zebrafish brains of 4/5 days postfertilization (dpf; see Baeuml et al., 2019; Biechl et al., 2016). These *shh* domains include classical floor plate cells defining the ventral midline of the neuraxis from spinal cord into the posterior diencephalon. However, the forebrain shows more complex *shh*-GFP expression patterns involving basal and alar plates. We will describe all *shh*-GFP expression domains from anterior to posterior levels, along with nuclear DAPI stains and, when necessary, with tyrosine hydroxylase (TH) immunostains. We use for identification of brain structures basically the *Neuroanatomy of the Zebrafish Brain* atlas (Wullimann, Rupp, & Reichert, 1996). In a recent study (Baeuml et al., 2019), we have detailed and justified some modifications from this atlas in the identification of the paraventricular organ, the intermediate hypothalamic nucleus and the posterior tuberal nucleus also applied here.

3.1 | Analysis of *shh*-GFP and tyrosine hydroxylase in transverse plane

3.1.1 | Telencephalon and preoptic region

Similar to the larval brain (4–8 dpf), there are no *shh*-GFP cell bodies in the adult subpallium and in the parenchyma of all adult pallial divisions. Different from the larval brain, however, radial glia cells (white arrows in Figure 1) in the adult medial zone of the dorsal telencephalon (Dm) stain for *shh*-GFP from precommissural (Figure 1a1–a2) via commissural (Figure 1b1–b2) to postcommissural levels (Figure 1c1–c2). These cells are identifiable by their soma location within the everted pallial ventricular surface and their long fibers extending towards the pial periphery (Figure 1d,d′). These fibers converge peripherally in the area identified as dorsal entopeduncular nucleus (ENd) and the ventral part of the posterior zone of the dorsal

TABLE 1 Antibodies

Antibody against	Host	Company	Dilution
Green fluorescent protein (GFP)	Chicken	Aves Labs # GFP-1020	1:500
Second	Donkey (Anti-chicken-FITC)	Dianova (Mol. Probes) #A11039 703-095155	1:100
Tyrosine hydroxylase (TH)	Mouse, monoclonal	Millipore (AbCys), #MAB318	1:100
Second	Donkey (Anti-mouse-Cy3)	Dianova, #715-166-151	1:400

telencephalon (Dp; Figure 1d) suggesting that this convergence area is a pial surface and not a ventricular surface in contrast to that of the medial (Dm), central (Dc) and lateral (Dl) zones of the dorsal telencephalon. The latter zone (Dl) shows no *shh*-GFP radial glia cells, but many other markers demonstrate the nature of its ventricular surface (see section 4).

In the postcommissural telencephalon, a strong *shh*-GFP expression domain is present in the most ventroposterior basal pallidal part of the subpallium (BP; Figure 1c,e). This area has not been identified as a separate entity before, including the *Neuroanatomy of the Zebrafish Brain* adult atlas (where it lies in the area between the anterior parvocellular preoptic nucleus, PPa, and the postcommissural nucleus of the ventral

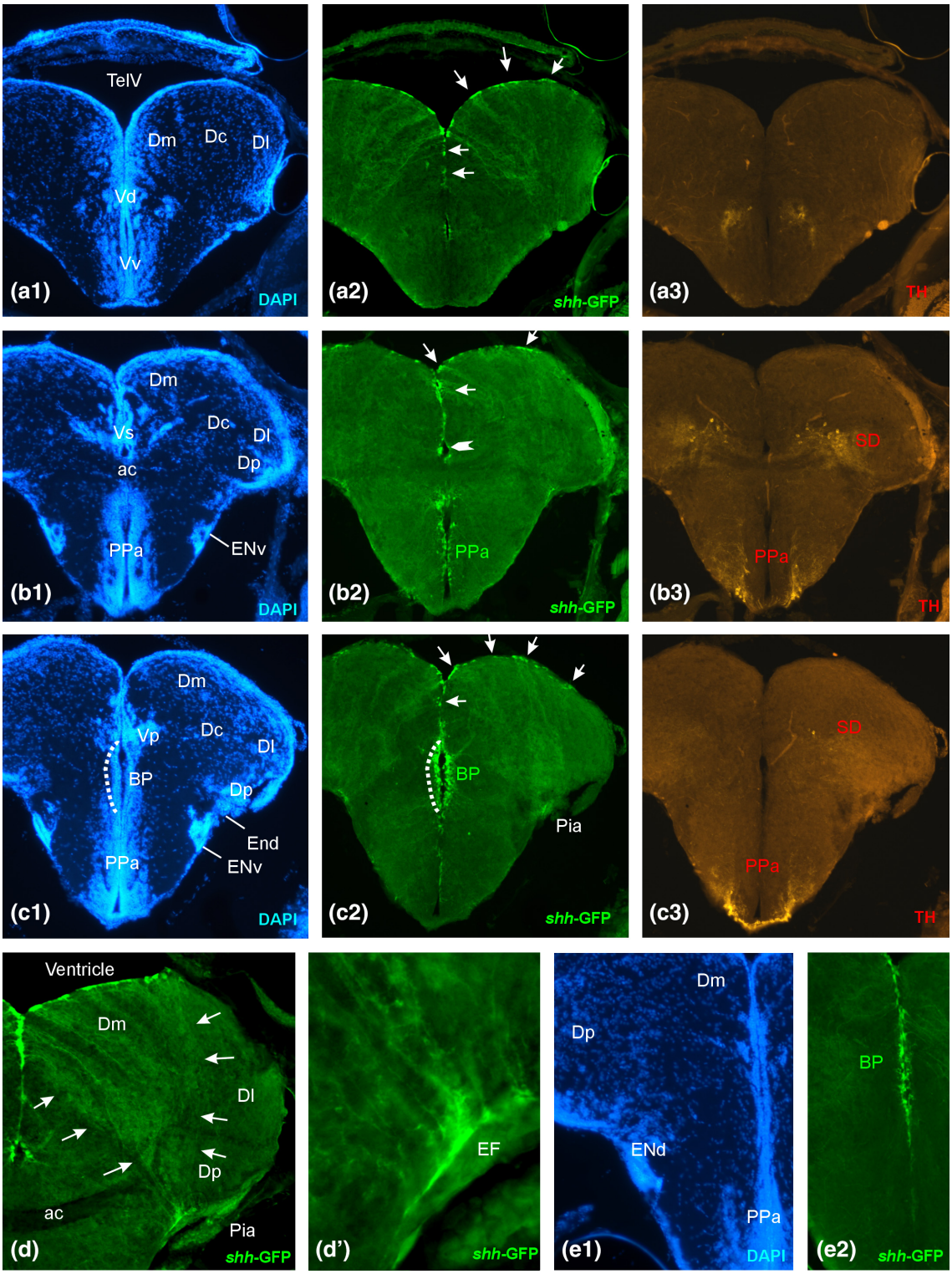


FIGURE 1 Legend on next page.

telencephalon, Vp; page 40, cross-section 107 in Wullimann et al., 1996). The spot of *shh*-GFP cells seen at the base of the supracommissural nucleus of the ventral telencephalon (Vs) is the most anterior extension of BP (arrowhead in Figure 1b2). We propose that this basal subpallial expression domain represents the zebrafish homolog of the mammalian pallidal *shh* expressing domain (Mueller & Wullimann, 2009; Mueller, Wullimann, & Guo, 2008). The *shh*-GFP cell somata in BP mostly do not lie directly at the ventricular lining and do not exhibit long fibers towards the pial periphery. Thus, they likely are not radial glia cells.

A fair number of *shh*-GFP cells is seen in the anterior parvocellular preoptic nucleus (PPa; Figure 1b–c) and a few cells show up in the suprachiasmatic nucleus, but none are present in the posterior parvocellular preoptic and magnocellular preoptic nuclei (SC, PPp, PM; Figures 2a,b and 3i,j). Furthermore, neither subpallial nor preoptic dopamine cell somata ever colocalize with *shh*-GFP (Figure 1a–c).

3.1.2 | Diencephalon

Transverse hind- and midbrain sections are leveled with respect to the caudorostral body axis and thus run horizontally through the forebrain because of the ventral bending of the neural tube's front end. As suggested previously (Herget, Wolf, Wullimann, & Ryu, 2014; their figure 1), it is reasonable to identify in such forebrain sections dorsal as posterior and ventral as anterior to avoid confusion with body axes. Thus, forebrain sections may show from "dorsal" (i.e., posterior) to "ventral" (i.e., anterior) at the same time parts of the (most posterior) prosomere 1 (pretectum; P1), prosomere 2 (thalamus, previously dorsal thalamus; P2), and prosomere 3 (prethalamus, previously ventral thalamus; P3) as well as the (most anterior) hypothalamus (Figures 2–4). It has to be noted that these prosomeres include alar as well as basal plate components (see below). This clarification of neural tube axes is critical in the present investigation that focuses on a ventrally expressed marker (*shh*) in order to keep attention to the true ventral versus dorsal side of the forebrain.

Numerous *shh*-GFP cell bodies are seen in the zona limitans intrathalamica (ZLI; Figures 2c–e and 3k) and in cells in the prethalamus, the alar plate of prosomere 3 (Figures 2a,b and 3j). This somewhat unexpected alar plate *shh*-GFP expression is paralleled by *islet1*-GFP expression (see section 4). The thalamus (Th) and periventricular pretectum (Pr; alar plate of prosomeres 2 and 1, respectively) contain no *shh*-GFP cell bodies. However, the periventricular pretectum exhibits *shh*-GFP positivity in terminal visual projection fibers (pretectal terminal field) originating in *shh*-GFP retinal ganglion cells (prtf; Figures 2c–e, 3k, and 4b) and similar thalamic retinal terminal fields are seen lateral to the (anterior) thalamus and prethalamus (thtf; Figures 2a,b and 3j).

Regarding diencephalic basal plate divisions, *shh*-GFP cell bodies clearly are present in both the parvocellular and magnocellular (pear-shaped) cells of the periventricular nucleus of the posterior tuberculum (TPp-p, TPp-m) as well as in the paraventricular organ (PVO; Figures 2c–e and 4a,b). The *shh*-GFP label in the periventricular posterior tuberculum is characterized by relatively far migrated cells of the TPp-m and peripherally leading labeled fibers. These fibers lead toward the preglomerular area where far migrated *shh*-GFP positive cell bodies are present (Figures 2e, 4b, and 5). Furthermore, *shh*-GFP cells are present in the dorsal and ventral periventricular hypothalamic zones (Hd, Hv; Figures 2d–f and 3k,l). In contrast, the adult caudal periventricular hypothalamic zone remains free of *shh*-GFP (Figures 3l, 4c, and 6a). However, in juvenile stages, Hc does express *shh*-GFP (see next section).

The *shh*-GFP cell bodies continue to be present in the basal plate domain of prosomere 1, which is the area of the nucleus of the medial longitudinal fascicle (Nmlf; Figures 2f and 6a). Most of these cells do not yet exhibit fibers typical for floor plate cells seen more posteriorly with the exception of some strongly stained midline cells (arrowhead in Figure 6) directly at the ventral midline ventricle. These *shh*-GFP cells have ventrally directed fibers as seen more posteriorly in the mid- and hindbrain and may be interpreted as most anterior floor plate cells. However, the remaining cells in the area of the Nmlf lie more distant to the ventricular lining and appear to lack such fibers. More "ventrally" (actually anteriorly because of the above mentioned neural tube bending) *shh*-GFP cells are seen in the TPp-m and the Hd (note inset in Figure 6a2).

3.1.3 | Colocalization of adult dopamine cells with *shh*-GFP

Because dopamine cells are present in the posterior tubercular nuclei mentioned above (TPp-p,TPp-m,PVO) and the posterior tuberal

FIGURE 1 Expression of *shh*-GFP and tyrosine hydroxylase in the adult zebrafish telencephalon. (a1–a3) Transverse section at precommissural telencephalic level shown in nuclear DAPI stain (a1), *shh*-GFP (a2) and tyrosine hydroxylase (TH; a3) immunostains. Note *shh*-GFP stained pallial radial glia cells (white arrows) in medial zone of dorsal telencephalon (Dm). (b1–b3) Transverse section at commissural telencephalic level shown in the same three stains. Note in addition to pallial radial glia cells (white arrows) some cells in the supracommissural nucleus of the ventral telencephalon (Vs; arrowhead) and more cells in the anterior parvocellular preoptic nucleus (PPa). (c1–c3) transverse section at postcommissural telencephalic level shown in same three stains. Note in addition to pallial radial glia cells also the distinct and compact *shh*-GFP stain in most posterior basal subpallium area (interpreted as basal pallidum [BP; enclosed by dashed line], previously unnamed in zebrafish, see text). Note that none of the *shh*-GFP cells are stained for TH at telencephalic levels (a3–c3). (d) Enlarged view of pallial radial glia cells (different specimen) and their fibers (enclosed by white arrows) which converge towards a ventrolateral pial surface where endfeet are formed. (d′) enlarged region of ventrolateral endfeet formed by pallial radial glia fibers originating in Dm. (e1–e2) Enlarged view of the location (e1) and *shh*-GFP immunostain (e2) in the BP (different specimen). See text for details. Abbreviations: ac, anterior commissure; BP, basal pallidum; Dc, Dm, Dl, Dp, central, medial, lateral, posterior zone of dorsal telencephalon; EF, endfeet; End/ENv, dorsal/ventral entopeduncular nucleus; PPa, anterior parvocellular preoptic nucleus; SD, subpallial dopaminergic cells; TelV, telencephalic ventricle; Vd/Vp/Vs/Vv, dorsal/postcommissural/supracommissural/ventral nucleus of ventral telencephalon [Color figure can be viewed at wileyonlinelibrary.com]

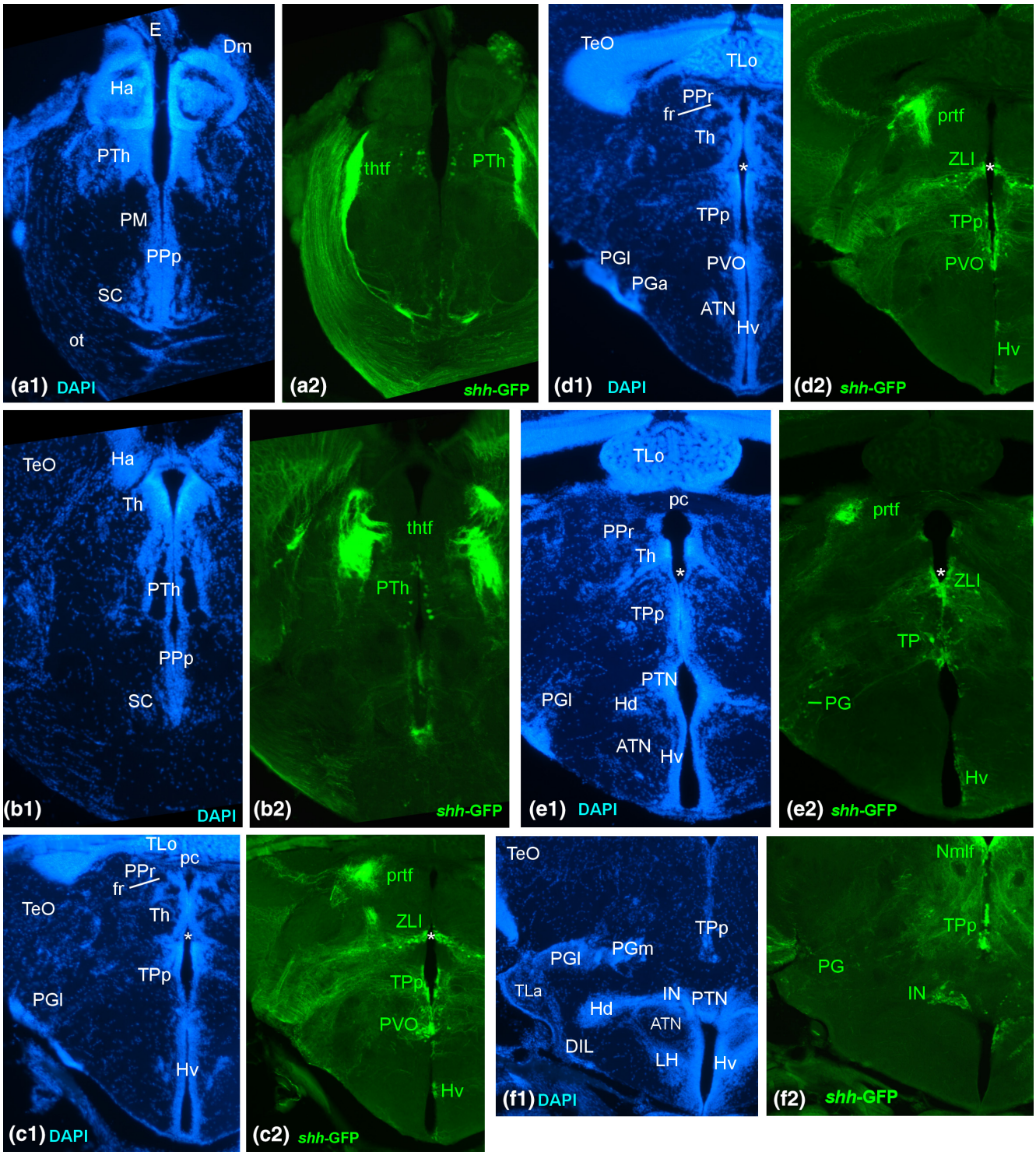


FIGURE 2 Expression of *shh*-GFP in the adult zebrafish diencephalon. Six transverse sections showing habenular/prethalamic (a), anterior thalamic (b), posterior thalamic (c), posterior tubercular/ paraventricular organ (d), posterior tuberal nuclear (PTN; e), and intermediate hypothalamic nuclear levels (f). The ventricle spot of the zona limitans intrathalamica is indicated with an asterisk. See text for details. Abbreviations: ATN, anterior tuberal nucleus; DIL, diffuse nucleus of lobus inferior; DiV, diencephalic ventricle; Dm, medial zone of dorsal telencephalon; E, epiphysis; fr, fasciculus retroflexus; Ha, habenula; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; IN, intermediate hypothalamic nucleus; Nmlf, nucleus of the medial longitudinal fascicle; ot, optic tract; pc, posterior commissure; PG preglomerular complex; PGa/PGl/PGm, anterior/lateral/medial nucleus of PG; PM magnocellular preoptic nucleus; PPp, posterior parvocellular preoptic nucleus; PPr, periventricular pretectum; prtf, pretectal retinal terminal field; PTh, prethalamus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; SC, suprachiasmatic nucleus; TeO, optic tectum; thtf, thalamic retinal terminal field; TLa, torus lateralis; TLo, torus longitudinalis; TPp, periventricular posterior tuberculum; ZLI, zona limitans intrathalamica [Color figure can be viewed at wileyonlinelibrary.com]

nucleus (PTN), an immunostain against tyrosine hydroxylase was applied to the same sections (Figure 4a3,b3,c3) and double-label with *shh*-GFP was indeed seen in three of four dopaminergic posterior tubercular systems (i.e., TPp-p,TPp-m,PVO; yellow arrows in Figure 4a,b; Table 2), but not in the adult PTN (Figure 4c). However, the juvenile PTN does stain for both *shh*-GFP and TH (see section 3.4). With the possible exception of the caudal zone of the periventricular hypothalamus, no other dopamine cells in the zebrafish brain show such double-label (i.e., in pretectum, prethalamus, or preoptic region). This is a stunning parallelism to the documented midbrain floor plate (i.e., *shh* expressing cells) origin of substantia nigra/ventral tegmental area in mammals and suggests that also diencephalic dopamine cells (including the striatal projecting cells) do originate from *shh* expressing cells (see section 4).

3.1.4 | Mesencephalon, rhombencephalon, and spinal cord

Posterior to the Nmlf, *shh*-GFP cell bodies are restricted to mesencephalic, rhombencephalic, and spinal floor plate cells situated in the ventral midline of the ventricular lining (Figure 6b–g). These cell bodies typically extend long fibers ventrally into the neural parenchyma which reach with their endfeet (EF) the ventral pial brain or spinal cord surface. The midbrain floor plate cell fibers are seen to bypass the oculomotor nerve on their passage towards the ventral pia (Figure 6b). The interpeduncular nucleus is invaded and surrounded by such fibers (Figure 6c). Noradrenergic cells of the locus coeruleus colocalize with *shh*-GFP (yellow arrows, Figure 6d), but other hindbrain catecholaminergic cells, such as the area postrema (Figure 6f) do not.

3.2 | Analysis of possible colocalization of *shh*-GFP and *islet1*-GFP

In the mid- and hindbrain, the *shh*-GFP cell bodies are restricted to floor plate cells and, thus, are never colocalized with *islet1*-GFP which is always localized in identifiable migrated brain nuclei, best studied for cholinergic motor neurons (see discussion in Baeuml et al., 2019). Because forebrain *shh*-GFP expression is more complex and includes many cell bodies beyond floor plate cells (sometimes peripherally migrated ones) we analyzed corresponding section levels of both *shh*-GFP (present study) and *islet1*-GFP (see also Baeuml et al., 2019) fish brains for the presence of possible co-localization of the two markers (Figure 3) in more detail. Generally, in brain structures positive for *shh*-GFP, these cell bodies are located directly at the ventricle with many more unstained cells peripheral to them (note dashed lines peripheral to *shh*-GFP cells in Figures 3g,h,k,l). In contrast, *islet1*-GFP cell bodies lie more peripheral leaving many unstained cells towards the ventricle in each area (note dashed lines towards ventricle respective to *islet1*-GFP cells in Figures 3a,b,e,f). Since we use two separate transgenic lines for this comparison, a double-label analysis is not directly possible in the same section. However, the distribution of the two transgenically expressed GFP markers is that mutually exclusive spatially as to make a colocalization extremely unlikely in the ventral telencephalon (Vs), preoptic region (PPa, SC), and dorsal and ventral zones of the periventricular hypothalamus (Hv, Hd). The more peripherally migrated magnocellular part of the periventricular posterior tubercular nucleus (TPp-m) and the paraventricular organ (PVO) are completely free of *islet1*-GFP cells (Baeuml, Biechl, & Wullimann, 2019). Although the (alar) prethalamic *shh*-GFP cells intermix with *islet1*-GFP cells, they are different cells because the former are never TH positive (this study) whereas *islet1*-cells are TH positive (Baeuml et al., 2019; see section 4). To decide about the very few *islet1*-GFP cells possibly positive for *shh*-GFP in the area of the Nmlf, a double transgenic line (*shh*-GFP and *islet1*-GFP) would be necessary. However, TH cells in the parvocellular part of the periventricular posterior tubercular nucleus (TPp-p) were found both to be *shh*-GFP positive (this study) and *islet1*-GFP positive. Thus, the TPp-p is the only nucleus with clear colocalization of both GFP markers.

3.3 | Analysis of *shh*-GFP and tyrosine hydroxylase in sagittal plane

In order to provide additional means of verification and didactically improved visualization of data reported above, we also prepared sagittal sections of *shh*-GFP transgenic brains immunostained against tyrosine hydroxylase.

An overview of a *shh*-GFP zebrafish brain (Figure 7a1)—when compared to its DAPI stained alter ego (Figure 7a2)—illustrates the complete absence of *shh*-GFP cell bodies in all parts of the cerebellum (valvula, corpus and lobus caudalis cerebelli) as well as in the primary sensory lateral line area (cerebellar crest and underlying MON) and chemosensory (facial and vagal) lobes and, furthermore, such negativity is noted in the diffuse nucleus of the inferior lobe, the caudal periventricular hypothalamus and corpus mamillare (Figure 7c2). In contrast, the *shh*-GFP positive line of midline floor plate cells in the entire hindbrain up to midbrain and into Nmlf is nicely visualized in sagittal view, including the ventrally directed fibers of these cells (Figure 7a1,b1,d). More anteriorly the zona limitans intrathalamica (ZLI) forms a *shh*-GFP positive spear-shaped transverse barrier between thalamus and prethalamus (Figure 7a1,b1) extending into the alar plate. A more focused higher-power sagittal analysis of the diencephalon shows the ZLI and its relationship to surrounding structures at various parasagittal levels (Figure 8a2–d2). Anterior to the ZLI, again basally located *shh*-GFP cells in the basal plate diencephalon (posterior tuberculum) and hypothalamus, as well as in the alar plate preoptic region and basal subpallium follow (PPa, BP; Figures 7a1,a2 and 8a2–d2). Turning to the posterior tuberculum, the striking large cells of the periventricular posterior tuberculum (TPp-m) are visualized to be double-labeled for TH and *shh*-GFP (yellow arrows; Figures 7b1–b3 and 8b–d). The higher-power diencephalic pictures also show that other diencephalic dopamine cell groups, such as the periventricular pretectum (Pr) and the prethalamic/zona incerta) cells

(PTh; Wullimann & Rink, 2001) or the preoptic nuclei (PPa, PPp, and SC) and the posterior tuberal nucleus (PTN) as well as the caudal periventricular hypothalamic zone (Hc) are remote from *shh*-GFP cells (Figure 8). Double-label of *shh*-GFP and TH in the parvocellular division of the periventricular tubercular nucleus (TPp-p) and in the paraventricular organ (PVO) are hard to visualize in the sagittal plane, but sufficiently documented above in transverse sections (compare Figure 4).

Overall, this sagittal analysis delivered a highly consistent and corroborating picture but also showed that certain details are veiled

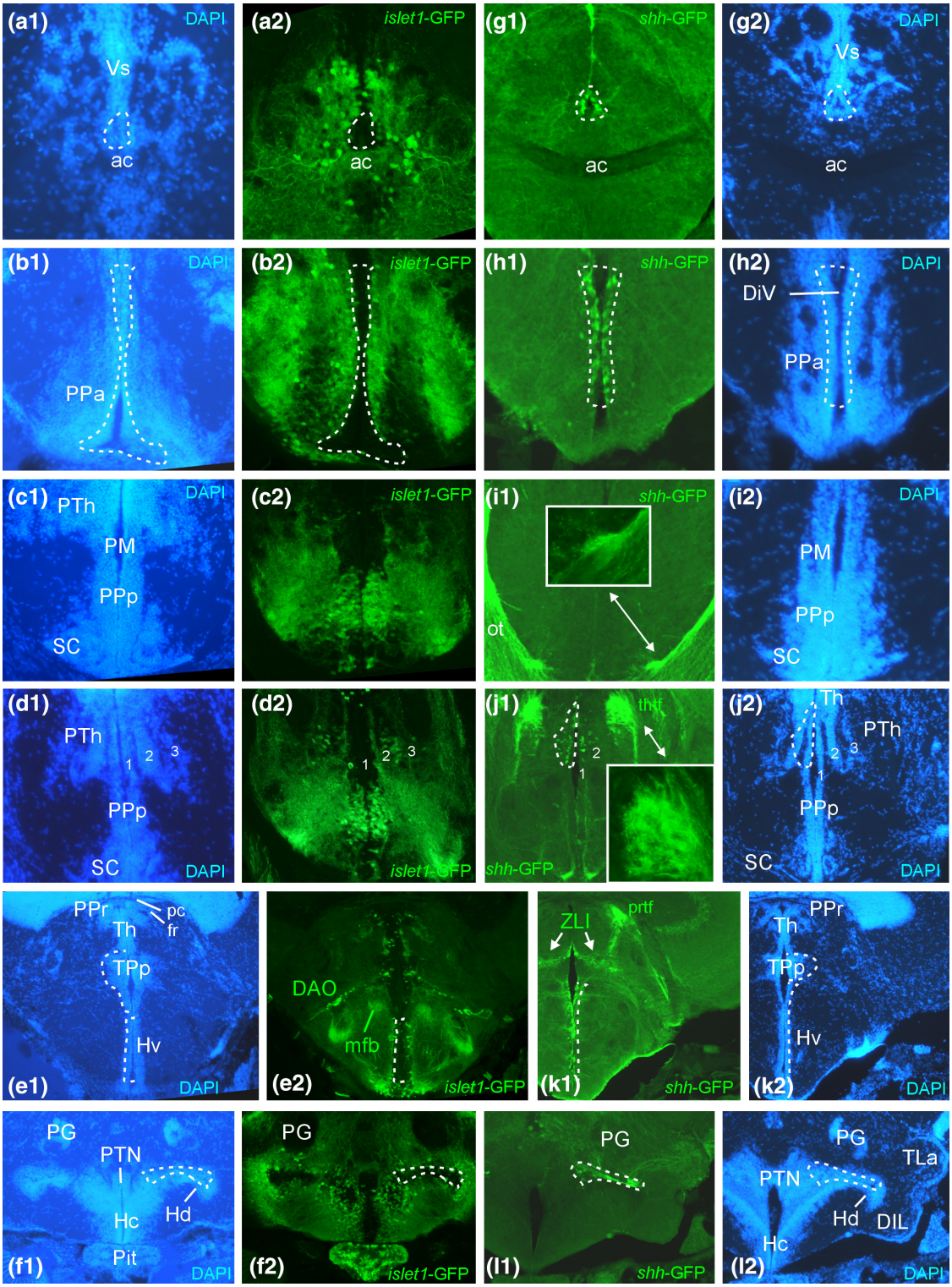


FIGURE 3 Legend on next page.

(double-labeled in TPp-p and PVO) whereas other facts are better visualized in sagittal sections (longitudinal distribution of floor plate cells and relationship of ZLI to thalamus and prethalamus). Also, certain most laterally placed *shh*-GFP cell groups were better to be grasped in transverse sections (preglomerular area, locus coeruleus, dorsal zone of periventricular hypothalamus; see above).

3.4 | Colocalization of late larval dopamine cells with *shh*-GFP

Because of the broad *shh*-GFP expression in early larval zebrafish brains (Baeuml et al., 2019; Biechl et al., 2016) in the posterior tuberculum and hypothalamus, we wanted to check later larvae with a progressed brain differentiation state for the presence of *shh*-GFP and TH. We focused particularly on the fourth posterior tubercular division (i.e., the posterior tuberal nucleus;PTN) and the caudal division of the periventricular hypothalamus (Hc) both showing a clear absence of *shh*-GFP but the presence of TH in the present study in adult brain sections. Also, this larval investigation allows for checking on a possible early emergence of the subpallial *shh*-GFP positive pallidal domain (BP).

At 13 dpf, the zebrafish diencephalon is already differentiated into its major divisions visualized in DAPI stains (Figure 9a1–e1), namely, the pretectum (Pr), thalamus (Th), prethalamus (PTh), preoptic region (Po), the area of the nucleus of the medial longitudinal fascicle (N), the posterior tuberculum (PT), the preglomerular region (PG), and the rostral (adult: ventral), intermediate (adult: dorsal) and caudal periventricular hypothalamus (Hr,Hi,Hc). The *shh*-GFP stain (Figure 9a2–e2) corroborates these divisions, for example, the position of the ZLI between thalamus and prethalamus and the broad *shh*-GFP expression in the basal plate of the midbrain tegmentum leading into N and PT and more anteriorly into Hr, Hi, and Hc. Furthermore, all larval zebrafish diencephalic dopamine groups visualized by TH (previously described by Rink and Wullimann (2002) are clearly visible at this stage, starting with the pretectal and preoptic groups leading to the parvocellular (adult: TPp-p; larval 1) and magnocellular divisions (adult TPp-m; larval 2,4) of the periventricular posterior tuberculum, and to the paraventricular organ (PVO, larval 3), the posterior tuberal nucleus (PTN, larval 6) and the caudal zone of the periventricular hypothalamus (Hc, larval 7; Figure 9a3–e3). Upon closer inspection of the latter two (Figure 9c–e), clear colocalization of *shh*-GFP and TH is seen in single large cells (yellow arrows in Figure 9) in the PTN (and, as in the adult, in the TPp-m), whereas most cells in the Hc appear single labeled for either *shh*-GFP or TH (white arrows in Figure 9).

We do not see at this stage a subpallial *shh*-GFP positive pallidal population (BP) as in the adult brain, and, thus, the preoptic cells (Figure 9a2) are the most anterior *shh*-GFP positive cells.

4 | DISCUSSION

4.1 | General issues: Expectations meet surprises

A comparison of early adult zebrafish brain *shh*-GFP expression (see Results and Figure 10a) with early larval expression (Baeuml et al., 2019; Biechl et al., 2016) reveals that, generally, expression domains are retained into the adult stage, starting posteriorly with longitudinally arranged series of floor plate cells in spinal cord, hindbrain, midbrain, and, most anteriorly, in the area of the nucleus of the medial longitudinal fascicle (Nmlf), that is, the basal plate division of the most posterior diencephalic prosomere (P1) representing the most posterior forebrain. The only additional *shh*-GFP cells in the hindbrain are a few noradrenergic locus coeruleus cells. Some *shh*-GFP cells in the Nmlf are the most anterior floor plate cells unmistakably characterized by long radial fibers that extend from their cell bodies at the midline ventricular floor toward the pial periphery where they form endfeet. However, the amniote floor plate itself appears to extend further anteriorly into the mammillary (caudal) hypothalamus as indicated by longitudinally expressed marker genes, such as the LIM homeobox

FIGURE 3 Analysis of colocalization of *shh*-GFP and *islet1*-GFP in the adult zebrafish brain. Transverse sections of an *islet1*-GFP brain are shown in two left columns (a–f) and of a *shh*-GFP brain in two right columns (g–l), both for GFP positivity and additionally shown in nuclear DAPI stain. The *islet1*-GFP data stem from our previous study (Baeuml et al., 2019). (a/g) supracommissural nucleus of ventral telencephalon (Vs). (b/h) anterior parvocellular preoptic nucleus (PPa). (c/i) suprachiasmatic nucleus (SC). (d/j) prethalamus (ventral thalamus; PTh). (e/k) periventricular nucleus of posterior tuberculum (TPp) and ventral zone of periventricular hypothalamus (Hv). (f/l) dorsal zone of periventricular hypothalamus (Hd). Generally, *shh*-GFP cells are located more ventricularly than *islet1*-GFP cells, as evidenced by dashed lines in Vs, PPa, Hv, and Hd where *shh*-GFP cells are always within the lining and *islet1*-GFP cells are on the outside (i.e., the latter are more distant from the ventricle than the former). For location of *shh*-GFP cells in TPp see Figure 4. While such ventricularly located *shh*-GFP cells are also seen in the prethalamus (tier 1 in d), both *shh*-GFP and *islet1*-GFP cells exist in tier 2 (or ventromedial thalamus; see section 4). In SC, the medial *islet1*-cells cells are far apart from the lateral *shh*-GFP signal. Inset in (i1) shows enlargement of *shh*-GFP cells in SC. Inset in (j1) shows enlargement of retinal terminal field lateral to the thalamus. See text for details. Abbreviations: ac, anterior commissure; DAO, dorsal accessory optic nucleus; DIL, diffuse nucleus of inferior lobe; DiV, diencephalic ventricle; fr, fasciculus retroflexus; Hc/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; mfb, medial forebrain bundle; pc, posterior commissure; PG, preglomerular complex; Pit, pituitary; PM, magnocellular preoptic nucleus; PPa/PPp, anterior/posterior parvocellular preoptic nucleus; PPr, periventricular pretectum; prtf, pretectal retinal terminal field; PTh, prethalamus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; SC, suprachiasmatic nucleus; TeO, optic tectum; Th, thalamus; thtf, thalamic retinal terminal field; TLa, torus lateralis; TPp, periventricular nucleus of posterior tuberculum; Vs, supracommissural nucleus of ventral telencephalon; ZLI, zona limitans intrathalamica; 1, ventricular layer of PTh; 2, ventromedial thalamic layer of PTh; 3, ventrolateral thalamic layer of PTh [Color figure can be viewed at wileyonlinelibrary.com]

gene *Lmx1b and the* forkhead gene *Fox1a* (Martínez, Puelles, Puelles & Echevarria, 2012; Puelles & Martínez, 2013; Puelles, Martínez-de-la-Torre, Bardet, & Rubenstein, 2012) and this might well be so in all vertebrates, including zebrafish.

Anterior to the Nmlf, adult *shh*-GFP cells continue to be present in zebrafish brain basal plate regions of thalamic and prethalamic prosomeres 2 and 3 (i.e., posterior tuberculum), as well as in the more anteriorly lying basal plate hypothalamus (dorsal and ventral zones of periventricular hypothalamus; Hd, Hv), and in the (alar plate) preoptic region. As in all vertebrates, the zebrafish zona limitans intrathalamica (ZLI) is visible as a transverse veil of cells forming a division between (dorsal) thalamus and prethalamus (ventral thalamus). The position of

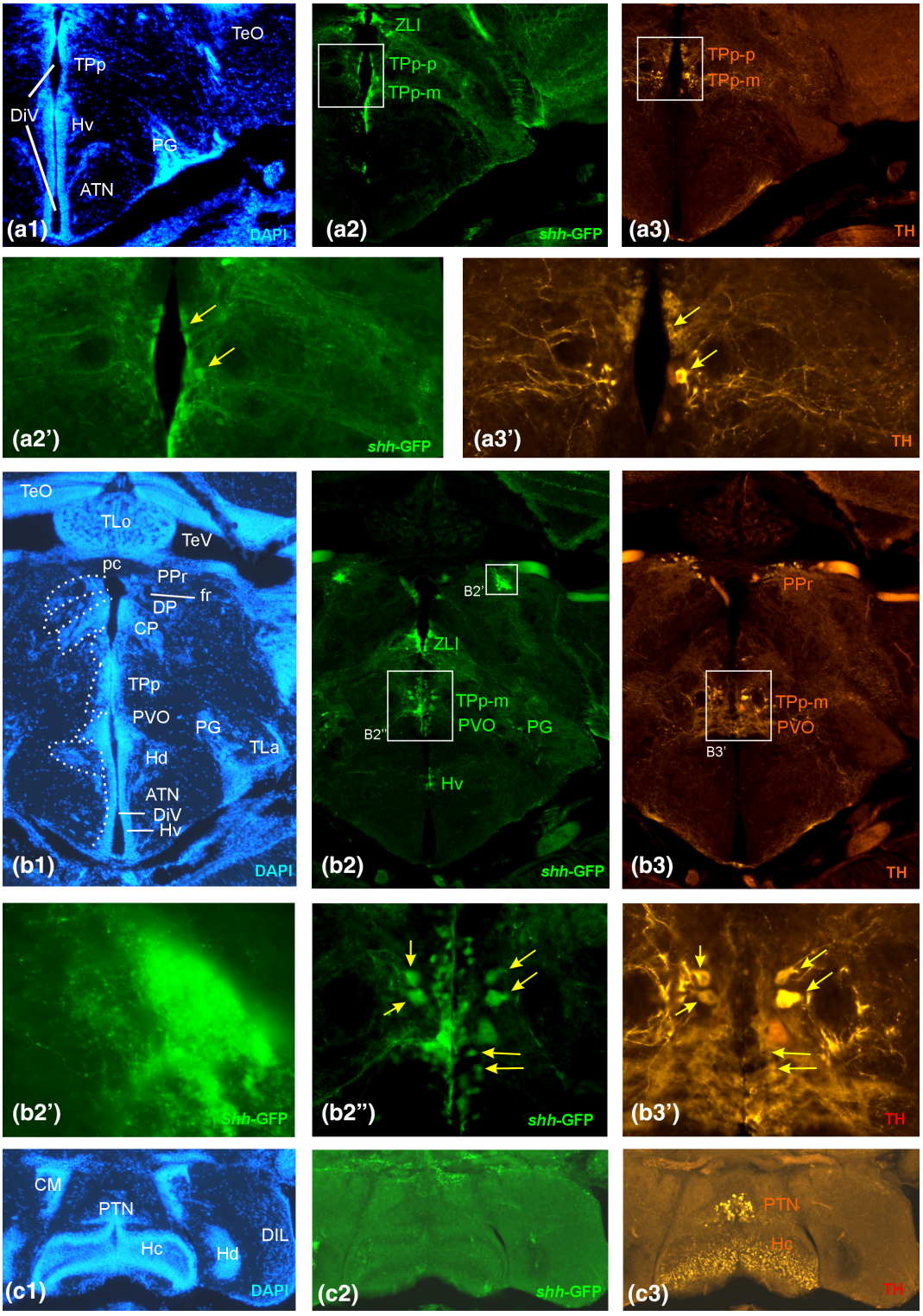


FIGURE 4 Legend on next page.

the ZLI is best seen in sagittal view in Figures 7b1 and 8a2–d2. Also the (alar) prethalamus contains *shh*-GFP cells (see section 4.3).

Transverse adult serial sections reveal that most zebrafish forebrain *shh*-GFP cell bodies lie close to the ventricle, yet they lack the typical cytological nature of floor plate cells seen more posteriorly. Only in a few forebrain regions are *shh*-GFP cell bodies observed in more migrated locations. This is expected for the zebrafish ZLI which forms a barrier between prosomeres 2 and 3 and, thus, its migrated cells are present in the diencephalic adult gray matter in this location (best seen in transverse view in Figure 2c2,d2). However, migrated *shh*-GFP cell bodies are also present in the area of the posterior tuberculum and, to a lesser degree, in the area of the nucleus of the medial longitudinal fascicle (Nmlf) as well as within the (alar plate) prethalamus. We will focus below on ventricularly located adult forebrain *shh*-GFP cells, and also consider peripherally migrated *shh*-GFP cells. All adult *shh*-GFP positive regions just mentioned are present in larval *shh*-GFP zebrafish brains in a less differentiated state as will also be discussed below (see sections 4.2–4.4).

However, we will start by discussing three more surprising findings of *shh* expression in the young adult zebrafish brain. First, all TH positive (i.e., dopamine) cells of the posterior tuberculum and many noncatecholaminergic preglomerular complex cells arise from diencephalic *shh*-GFP cells (see section 4.2). Second, a *shh*-GFP positive basal pallidal population is identified in the zebrafish brain which almost certainly corresponds to the ventral telencephalic *shh* signaling center known in amniotes to guide telencephalic dorsoventral development (sections 4.3.1–4.3.3). Third, a population of *shh*-GFP positive radial glia cells is newly detected in the adult zebrafish telencephalon (see section 4.3.4).

4.2 | Analysis of tyrosine hydroxylase and *shh*-GFP suggests that posterior tubercular dopamine cells and preglomerular cells arise from *shh*-GFP cells

It is widely accepted that a continuum of dopamine (i.e., tyrosine hydroxylase expressing) cells extends in embryonic amniote brains from the midbrain floor into basal diencephalic territories up to P3 (Puelles & Verney, 1998; Vitalis, Cases, Engelkamp, Verney, & Price, 2000; Smeets & González, 2000; Björklund & Dunnett, 2007; Smidt & Burbach, 2007; Smits, Burbach, & Smidt, 2006; Smits, von Oerthel, Hoekstra, Burbach, & Smidt, 2013; review in Wullimann, 2014). These mesodiencephalic dopamine cells will form the adult substantia nigra pars compacta (SN) and the ventral and lateral tegmental area (VTA/LTA) classically interpreted to be solely mesencephalic (Nieuwenhuys, 1985). Embryonically, these dopamine cells are described to arise mostly from midbrain floor plate (Verney, 1999). Moreover, HNF3ß, a floor plate marker induced by notochordal SHH (Echelard et al., 1993) is also seen in dopamine neurons of SN/VTA and, importantly, of basal diencephalon, as HNF3ß coincides with TH expression there and the same is seen in adults (Thuret, Bhatt, O'Leary, & Simon, 2004). Furthermore, it has been demonstrated that mesodiencephalic SN/VTA dopamine cells arise from the ventralmost neuroepithelium positive only for *shh* (and not for *Nkx2.2*, a more dorsally longitudinally expressed marker gene; Puelles et al., 2004). Finally, fate studies completed the picture by clearly showing that all divisions of the mesodiencephalic midbrain dopamine cells arise directly from *shh* expressing cells (Blaess et al., 2011; Hayes et al., 2011; Joksimovic et al., 2009).

Our findings in transgenic *shh*-GFP zebrafish brains reveal a striking parallel to this midbrain floor plate and ventral diencephalic *shh* cell origin of SN/VTA dopamine cells in amniotes. Teleosts lack midbrain dopamine cells, but exhibit such cells in the ventral diencephalon (posterior tuberculum; reviewed in Smeets & Reiner, 1994a, 1994b; Smeets & González, 2000; Wullimann, 2014). These ventral diencephalic dopamine cells include projection cells to the teleostean striatum (Mueller et al., 2008; Rink & Wullimann, 2001). Our *shh*-GFP data show that among all dopamine neurons in the adult zebrafish brain, exclusively those in the posterior tuberculum are *shh*-GFP positive (compare Figure 4 and Table 2). This is the case for the parvocellular (TPp-p) and magnocellular (TPp-m; i.e., the striatal projection neurons) nuclei of the periventricular posterior tuberculum and for the paraventricular organ (PVO), strongly suggesting that these dopaminergic posterior tubercular cells are direct derivatives of ventral diencephalic *shh* cells. Furthermore, the fourth posterior tubercular dopaminergic nucleus, the posterior tuberal nucleus (PTN), does also coexpress on the cellular level *shh*-GFP and TH at larval stages (although it loses *shh*-GFP in the adult brain). Other zebrafish dopamine cells in prethalamus, pretectum, preoptic region, telencephalon, and likely also hypothalamic ones (but see below), do not show such a colocalization.

FIGURE 4 Expression of *shh*-GFP and tyrosine hydroxylase in the adult zebrafish posterior tuberculum. Two consecutive and one more posterior transverse section are shown each for nuclear DAPI stain (a1,b1,c1), *shh*-GFP (a2,b2,c2) and tyrosine hydroxylase (TH; a3,b3,c3) immunostains. Enlargements demonstrate neurons double-labeled for *shh*-GFP and TH in the parvocellular and magnocellular parts of the periventricular posterior tubercular nucleus (yellow arrows; TPp-p, TPp-m; a2′,a3′), as well as in the TPp-m and the paraventricular nucleus (PVO; yellow arrows; b2″,b3′), but such double-label is absent in the posterior tuberal nucleus (PTN) and the caudal periventricular hypothalamic zone (Hc; c1–c3). (b2′) Enlargement of *shh*-GFP signal in retinal projection field lateral to the periventricular pretectal nucleus. See text for details. Abbreviations: ATN, anterior tuberal nucleus; CM, corpus mamillare; CP, central posterior thalamic nucleus; DIL, diffuse nucleus of lobus inferior; DiV, diencephalic ventricle; DP, dorsal posterior thalamic nucleus; fr, fasciculus retroflexus; Hc/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; pc, posterior commissure; PG, preglomerular complex; PPr, periventricular pretectum; PTN, posterior tuberal nucleus; PVO, paraventricular organ; TeO, optic tectum; TeV, tectal ventricle; TLa, torus lateralis; TLo, torus longitudinalis; TPp, periventricular nucleus of posterior tuberculum; TPp-p, parvocellular part of TPp; TPp-m, magnocellular (pear-shaped) cell part of TPp [Color figure can be viewed at wileyonlinelibrary.com]

Thus, this coexpression of *shh*-GFP and TH in posterior tubercular nuclei is a striking developmental parallel to the amniote mesodiencephalic dopamine cells of SN/VTA and supports homology of the teleostean posterior tubercular striatal projecting neurons with the diencephalic portion of the amniote SN/VTA.

The finding that these posterior tubercular dopamine cells may derive directly from *shh* expressing cells is further consistent with the recently reported *islet1*-GFP patterns in the adult zebrafish brain (Baeuml et al., 2019). While *islet1* is expressed in TPp-p, it is completely absent in TPp-m, PVO and PTN despite a strong larval *shh*

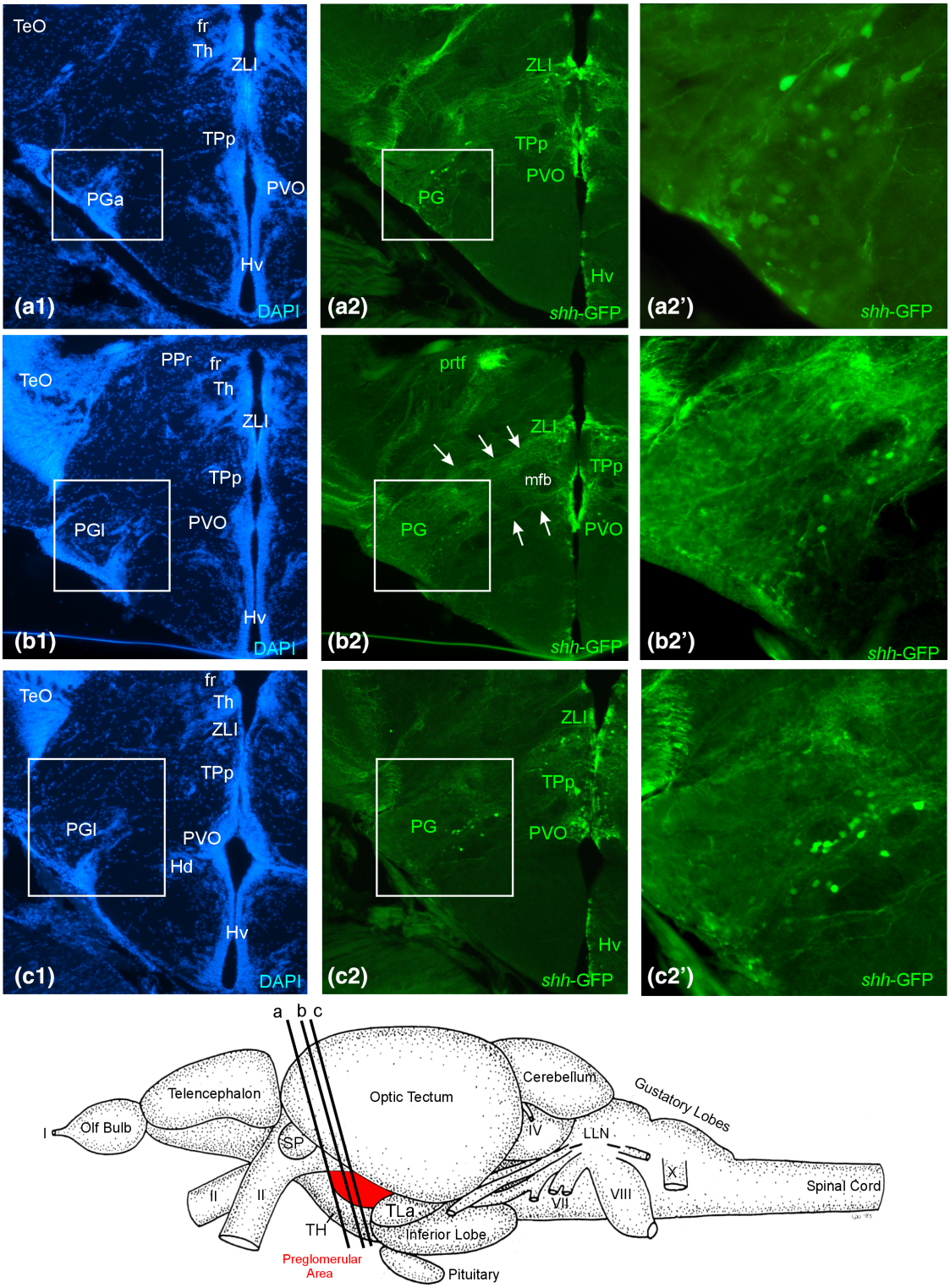


FIGURE 5 Legend on next page.

expression there, making it unlikely that dopamine cells there are induced there via *islet1* expression. However, in TPp-p, dopamine cells do colocalize with *islet1*-GFP (Baeuml et al., 2019) and with *shh*-GFP (this study) and, thus, these *shh* expressing cells might transform into dopamine cells which express *islet1*.

While dopamine cells are seen in the adult posterior tuberal nucleus (PTN) and caudal zone of periventricular hypothalamus (Hc), *shh*-GFP is absent there. However, as mentioned above already, larvae (Figure 9 (13dpf)) additionally show double-label of *shh*-GFP and TH in PTN. Although most cells in Hc were only single-labeled for either *shh*-GFP or TH (Figure 9) we cannot exclude double-label. Moreover, dopamine cells are not visualized by conventional antibodies in the intermediate nucleus of the periventricular zone of the dorsal periventricular hypothalamus and the anterior part of the caudal zone of the periventricular hypothalamus (Yamamoto et al., 2010, 2011). Therefore, an origin from *shh* expressing cells may be common for all divisions of zebrafish posterior tubercular and hypothalamic dopamine cells. Possibly, the exposure of *shh*-GFP fish to cyclopamine—which interferes with SHH function—would be a way to check on resulting absence of *islet1*-GFP cells and selective absence of certain dopamine groups (i.e., posterior tuberculum, but not prethalamus or pretectum).

Finally, beyond these posterior tubercular dopamine groups, a second population of differentiated zebrafish brain neurons that expresses *shh*-GFP can clearly be identified in the preglomerular complex (Figure 5). This large migrated diencephalic area is specific for teleosts and involved in processing ascending sensory information (reviews: Wullimann, 1998, Wullimann & Mueller, 2004; Wullimann & Grothe, 2013). It contains neither monoaminergic nor cholinergic cell bodies (review: Mueller & Wullimann, 2016) but contains few GABAergic and arguably many more glutamatergic cells (Mueller, Wullimann, & Guo, 2008; Mueller & Wullimann, 2016). The preglomerular *shh*-GFP cells are mostly located in the more anterior division of the PG and seem to have migrated out along radial fibers from the periventricular posterior tuberculum. Thus, the posterior tubercular dopamine cells together with these preglomerular neurons represent two clear cases in the zebrafish forebrain for which a direct origin from *shh* expressing cells is suggestive. In the adult zebrafish hypothalamus, we only see *shh*-GFP cells within the periventricular zones close to the ventricle and none in migrated areas. However, considerable numbers of mammalian hypothalamic (preoptic, tuberal, and mammillary) nuclei have been shown in elegant mouse brain fate studies to arise from *Shh*-expressing cells (Alvarez-Bolado, Paul, & Blaess, 2012).

4.3 | Newly identified adult *shh*-GFP cells in zebrafish basal pallidal region (BP) and in pallial radial glia cells (Dm; pallial amygdala)

In tetrapods, as particularly well studied in amniotes, a basal telencephalic (pallidal) *sonic hedgehog* (*shh*) expressing center is known to be instrumental for correct basal ganglia development and telencephalic dorsoventral patterning in general (for citations see section 4.3.2 and 4.3.3). However, *shh* expression in the larval zebrafish telencephalon remains elusive in the literature. A fine spot of *shh* expression in the embryonic zebrafish telencephalon has sometimes been claimed (e.g., by Krauss et al., 1993, at 26 hr; their figure 2F; or Ertzer et al., 2007, at 42 hr; their figure 6A). However, Holzschuh et al. (2003), Ekker et al. (1995), and Strähle et al. (1996) all did not note a telencephalic *shh* expressing population, and it remained unclear in Hagemann and Scholpp (2012). Major reviews on the subject of a pallidal *shh* signaling center (e.g., Wilson & Rubenstein, 2000) only speak about amniotes. Thus, in the previous literature, there is no clear indication of a *shh* expression domain in the zebrafish ventral telencephalon. Therefore, we re-examined this issue in the established *shh*-GFP zebrafish line during larval stages between 2 and 13 days and in adult (3-month-old) brains. We definitely see no *shh*-GFP cell bodies in the telencephalon up to 13 dpf larvae. The most anterior *shh*-GFP cells of the larval zebrafish are located in the preoptic region, in line with *in situ* hybridization studies cited above.

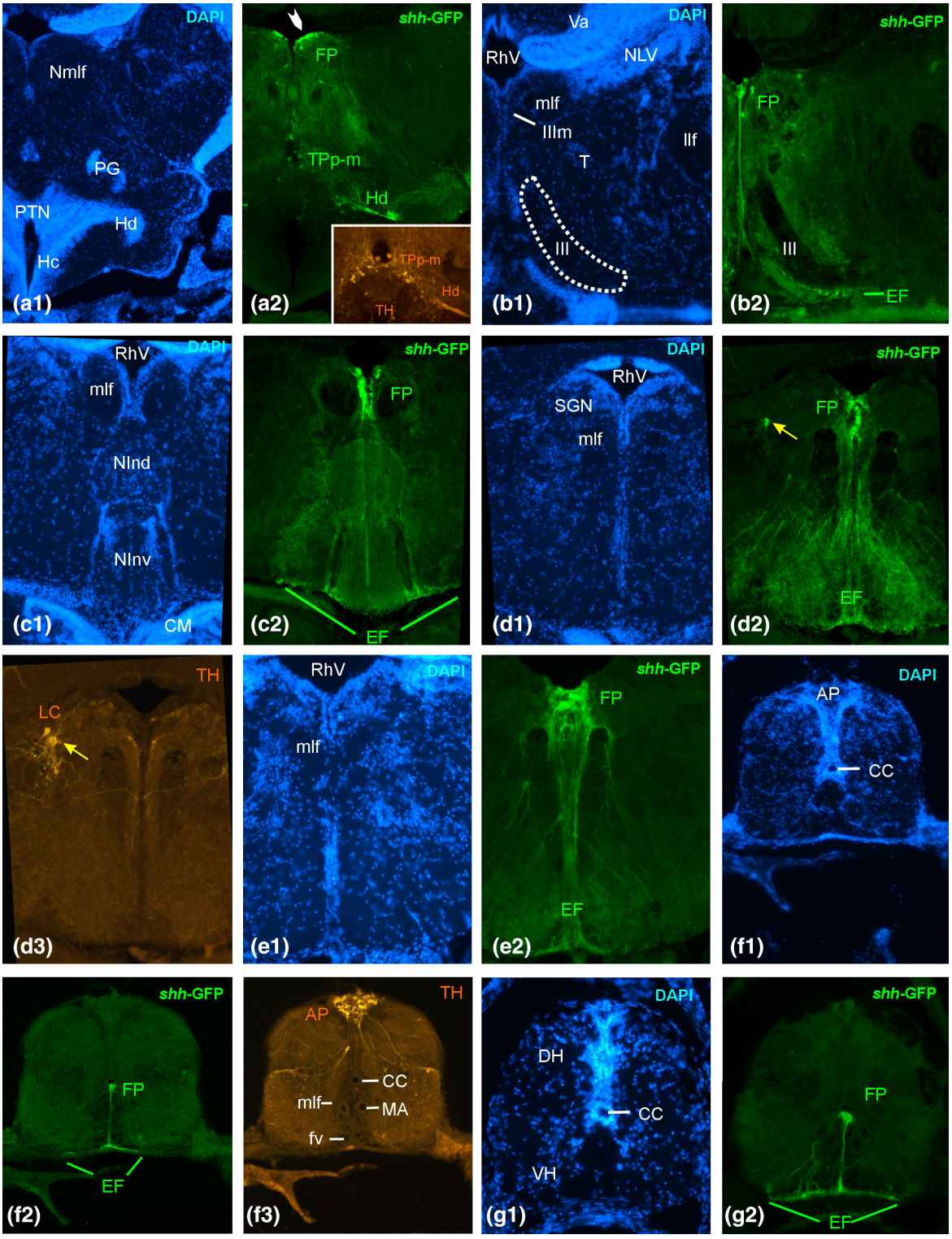
However, in the early adult zebrafish brain, two additional telencephalic groups of *shh*-GFP cells are newly seen. The first is a most posterior basal subpallial (pallidal) population (BP) which somewhat extends into basal telencephalic ventral nuclei (supracommissural and postcommissural nuclei of the ventral telencephalon; Vs, Vp). The second population is represented by *shh*-GFP positive radial glial cells at the ventricular lining of the medial zone of the dorsal telencephalon (Dm, pallial amygdala; Portavella, Vargas, Torres, & Salas, 2002; Portavella, Torres, & Salas, 2004; Wullimann & Mueller, 2004; Lal et al., 2018).

4.3.1 | Basal pallidal *shh*-GFP population

We detect in early adult zebrafish brains in the intermediate area between the anterior parvocellular preoptic nucleus (PPa) and the ventral telencephalic (subpallial) supracommissural (Vs)/posterior (Vp) nuclei an area which contains a distinct population of *shh*-GFP labeled cell bodies (BP; Figure 1). This area has remained

FIGURE 5 Expression of *shh*-GFP in the preglomerular complex. Three transverse sections (levels shown in schema at bottom) from anterior (a) to caudal (c) preglomerular complex levels. Note that fibers (white arrows in b2) originating in *shh*-GFP positive periventricular cells of the posterior tuberculum (TP) extend towards the preglomerular complex (PG) and that numerous *shh*-GFP cell bodies in far peripherally migrated positions are contained in the PG as detailed in enlargements (a2′–c2′). Note that in these enlargements, the *shh*-GFP positive fibers in upper left corner are retinal fibers terminating in the optic tectum. See text for details. Abbreviations: fr, fasciculus retroflexus; Hd/Hv, dorsal/ventral zone of periventricular hypothalamus; LLN, lateral line nerves; mfb, medial forebrain bundle; Olf, olfactory; PG, preglomerular complex; PGa, anterior nucleus of PG; PGl, lateral nucleus of PG; PPr, periventricular pretectum; prtf, pretectal retinal terminal field; PVO, paraventricular organ; SP, superficial pretectum; TeO, tectum opticum; Th, thalamus; TH, tuberal hypothalamus; TLa, lateral torus; TPp, periventricular nucleus of posterior tuberculum; ZLI, zona limitans intrathalamica; I, olfactory nerve; II, optic nerve; IV, trochlear nerve; VII, facial nerve; IX, glossopharyngeal nerve; X, vagal nerve [Color figure can be viewed at wileyonlinelibrary.com]

neuroanatomically uncharted in the *Neuroanatomy of the Zebrafish Brain* (see Wullimann et al., 1996; p. 40, cross-section 107). The *shh*-GFP cells continue somewhat into the basal posterior (Vp) and supracommissural (Vs) nuclei of the ventral telencephalon and maybe slightly even into the ventral part of the dorsal nucleus which represents the differentiated pallidum (Vdv; Mueller et al., 2008; Mueller & Wullimann, 2009). In any case, this *shh*-GFP population is a basal telencephalic domain located in the ventroposterior subpallium, that is, basal pallidum (BP). This is the first time that the telencephalic *shh* signaling center in the basal pallidum common to



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all tetrapods has unequivocally been visualized in the zebrafish brain.

4.3.2 | The comparative and developmental significance of the basal pallidal *shh* domain

Expression studies of longitudinally expressed genes are fundamental for the understanding of the vertebrate neural tube ventralization process (Figure 10b,c). In a seminal paper, Shimamura, Hartigan, Martínez, Puelles, and Rubenstein (1995) conceptually summarized and extended information on amniote *Shh* and related expression studies (Echelard et al., 1993; Ericson, Muhr, Jessel, & Edlund, 1995; Ericson, Muhr, Placzek, et al., 1995; Lazzaro, Price, de Felice, & di Lauro, 1991; Martí, Takada, Bumcrot, Sakaki, & McMahon, 1995; Placzek, Jessell, & Dodd, 1993; Placzek, Tessier-Lavigne, Yamada, Jessell, & Dodd, 1990; Price et al., 1992; Roelink et al., 1994; Van Straaten, Hekking, Wiertz-Hoessels, Thors, & Drukker, 1988; Yamada, Pfaff, Edlund, & Jessell, 1993) and described *Shh* expression in floor plate of spinal cord and hindbrain and its lateral expansion into the basal plate of the midbrain.

TABLE 2 Adult zebrafish brain nuclei with *shh*-GFP with/without tyrosine hydroxylase (TH). [Color table can be viewed at wileyonlinelibrary.com]

Structure	*shh*-GFP	TH	Colocalization *shh*-GFP/TH
Medial zone of dorsal telencephalon (Dm) (radial glia)	(+)	–	N.A.
Basal supracommissural nucleus of ventral telencephalon (Vs) (midline)	(+)	–	N.A.
Caudal subpallium (midline)	+	–	N.A.
Anterior parvocellular preoptic nucleus (PPa)	+	+	No
Posterior parvocellular preoptic nucleus (PPp)	–	+	N.A.
Magnocellular preoptic nucleus (PM)	–	(+)	N.A.
Suprachiasmatic nucleus (SC)	+	(+)	No
Zona limitans intrathalamica	+	–	N.A.
Ventral thalamus (VT,~Zona incerta)	+	+	No
Small cells of periventricular posterior tubercular nucleus (TPp-p)	+[1]	+	Yes
Large cells of periventricular posterior tubercular nucleus (TPp-m)	+	+	Yes
Paraventricular organ (PVO)	+	+	Yes
Posterior tuberal nucleus (PTN)[a]	+[a]	+[a]	Yes
Nucleus of medial longitudinal fascicle (Nmlf)	+[2]	–	N.A.
Ventral zone of periventricular hypothalamus (Hv)	+	–	N.A.
Dorsal zone of periventricular hypothalamus (Hd)	+	–	N.A.
Intermediate hypothalamic nucleus (IN)	+	–[b]	N.A.
Posterior part of caudal zone of periventricular hypothalamus (Hc)[a]	+[a]	+[a]	May be
Locus coeruleus (LC)	+	+	Yes

Notes: (+) few cells. *Red*: Dopamine/noradrenaline systems with suggested direct origin from *shh* expressing cells (see text and Figure 4) with 1 representing only case for TH cells colocalized with *islet1*-GFP (see text). *Blue*: Potential *shh*- and *islet1*-GFP colocalization ruled out (see text and Figure 4) with 2 being (unlikely) exception.

[a]observed only in 13d zebrafish brains, where *shh*-GFP and TH are colocalized in PTN cells and maybe in posterior Hc.

[b]Note that these cells express *TH2*—not visualized with TH antibodies—and contain dopamine (Yamamoto, Ruuskanen, Wullimann, & Vernier, 2011) and may thus potentially be double-labeled with *shh*-GFP.

FIGURE 6 Floor plate *shh*-GFP and tyrosine hydroxylase expression in adult zebrafish brain. Transverse sections run from posterior diencephalon (a: Prosomere 1, nucleus of the medial longitudinal fascicle; Nmlf) through midbrain (b: Oculomotor nerve nucleus; NIII; the corresponding nerve III is surrounded with dashed line) and hindbrain (c: Level of interpeduncular nucleus; NIn; d: Level of locus coeruleus; LC; e: Posterior hindbrain; f: Level of area postrema: AP) down to spinal levels (g). Note that fibers of the *shh*-GFP floor plate cells extend towards the pial periphery where they form endfeet (EF). In order to allow for identification of the magnocellular periventricular posterior tubercular nucleus, its tyrosine hydroxylase positive cells are shown in inset in (a2). Note that some noradrenergic locus coeruleus cells colocalize with *shh*-GFP label (yellow arrows), but not other hindbrain catecholaminergic cells (see text for details). Abbreviations: AP, area postrema; CC, central canal; CM, corpus mamillare; DH, dorsal horn; EF, radial glia endfeet; FP, floor plate; fv, funiculus ventralis; Hc/Hd, caudal/dorsal zone of periventricular hypothalamus; LC, locus coeruleus; llf, lateral longitudinal fascicle; MA, Mauthner axon; mlf, medial longitudinal fascicle; NInd/NInv, dorsal/ventral interpeduncular nucleus; Nlmf, nucleus of the medial longitudinal fascicle; NLV, nucleus lateralis valvulae; PG, preglomerular complex; PTN, posterior tuberal nucleus; RhV, rhombencephalic ventricle; SGN, secondary gustatory nucleus; T, midbrain tegmentum; TPp-m, magnocellular (pear-shaped) cell part of TPp; Va, valvula cerebelli; VH, ventral horn; III, oculomotor nerve; IIIm, oculomotor nerve nucleus [Color figure can be viewed at wileyonlinelibrary.com]

Anterior to the midbrain, a morphologically defined floor plate is no longer seen, but *Shh* is still expressed in basal plate diencephalon (P1–P3) through the hypothalamus up into the (alar) preoptic area. A vertebrate typical deviation from this longitudinal course is seen at the interface of thalamus (P2) and prethalamus (P3) where *Shh* extends in transverse direction dorsally (Figure 10c). All along its neuraxial course from spinal cord up to preoptic levels, the *Shh* expression domain is closely accompanied dorsally by a thinner expression stripe of the homeobox gene *Nkx2.2* (Price et al., 1992; Qiu et al., 1998; Shimamura et al., 1995). The related *Nkx2.1* gene is exclusively expressed in the forebrain, largely overlapping with *Shh* from P3 into hypothalamus and preoptic region (Figure 10c; Lazzaro et al., 1991; Shimamura et al., 1995; Kimura et al., 1996; Qiu et al., 1998). Furthermore, ventral forebrain *Islet1* expressing cells coexpress *Nkx2.1* (Ericson, Muhr, Placzek, et al., 1995) in contrast to posterior *islet1* cells (where *Nkx2*.1 is not expressed). In lateral views, the basal telencephalon appears to form an upper floor of *Shh* expression. However, transverse views reveal that *Shh* has a continuous expression in the neural wall from the preoptic area (POA) into the so-called anterior entopeduncular area (AEP) which in turn continues dorsally into the most basal division of the medial ganglionic eminence (Figure 10B2; MGE, i.e., the future pallidum; Asbreuk et al., 2002; Bulfone et al.,

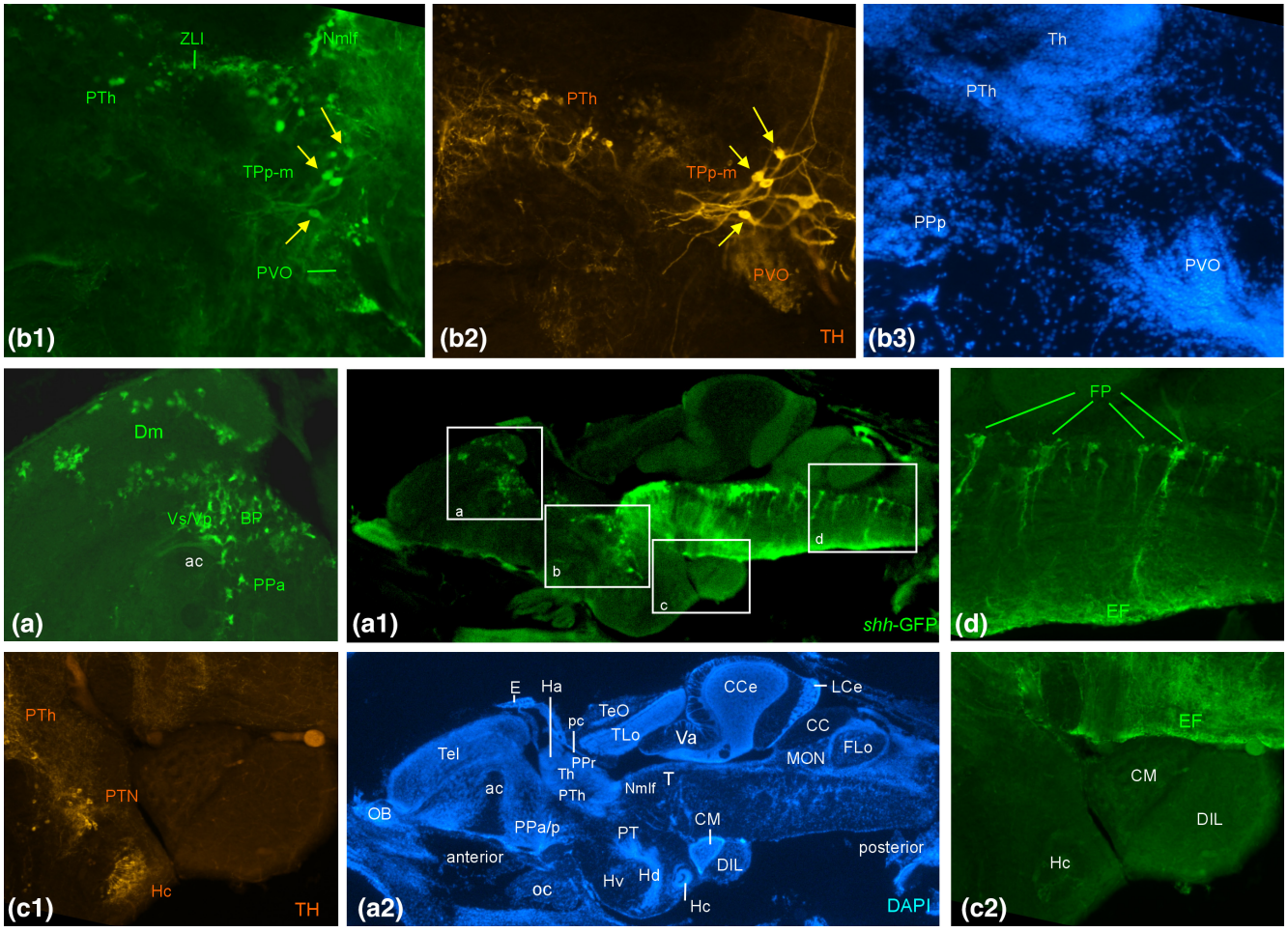


FIGURE 7 Sagittal analysis: Overview of *shh*-GFP and tyrosine hydroxylase expression in adult zebrafish brain. Parasagittal section shown for *shh*-GFP immunostain (a1) and shown for nuclear DAPI stain (a2). Enlargements (frames a through d in a1) show telencephalon (a), posterior tuberculum (b1–b3), hypothalamus (c1–c2) and hindbrain (d) in these two stains plus tyrosine hydroxylase (TH) immunostains when appropriate. Note that all *shh*-GFP cells in pallium are radial glia cells at the wrinkled medial and dorsal surface of the medial zone of the dorsal telencephalon (compare with transverse sections in Figure 1). Yellow arrows: Colocalization of *shh*-GFP and TH in magnocellular cells of periventricular posterior tuberculum (TPp-m). See text for details. Abbreviations: ac, anterior commissure; BP, basal pallidum; CC, crista cerebellaris; CCe, corpus cerebelli; CM, corpus mamillare; DIL, diffuse nucleus of lobus inferior; Dm, medial zone of dorsal telencephalon; EF, endfeet; FLo, facial lobe; FP, floor plate; Hc/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; LCe, lobus caudalis cerebelli; MON, medial octavolateralis nucleus; Nmlf, nucleus of the medial longitudinal fascicle; OB, olfactory bulb; pc, posterior commissure; PPa/p, anterior/posterior parvocellular preoptic nucleus; PPr, periventricular pretectum; PT, posterior tuberculum; PTh, prethalamus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; T, midbrain tegmentum; Tel, telencephalon; TeO, optic tectum; TeV, tectal ventricle; Th, thalamus; TLo, torus longitudinalis; TPp-p, parvocellular cell part of periventricular posterior tubercular nucleus; TPp-m, magnocellular (pear-shaped) cell part of periventricular posterior tubercular nucleus; Va, valvula cerebelli; Vp/Vs, postcommissural/supracommissural nucleus of ventral telencephalon; ZLI, zona limitans intrathalamica [Color figure can be viewed at wileyonlinelibrary.com]

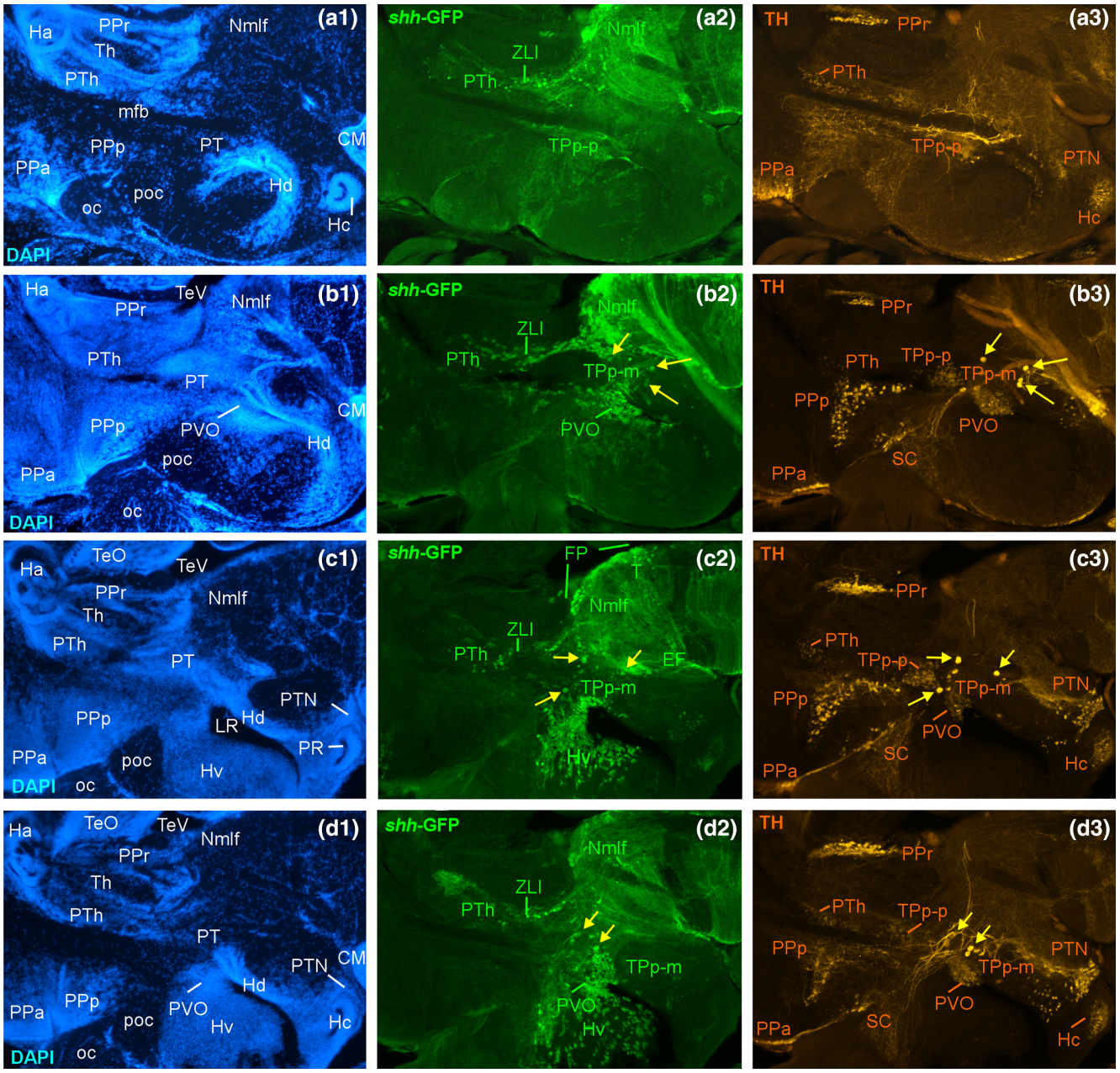
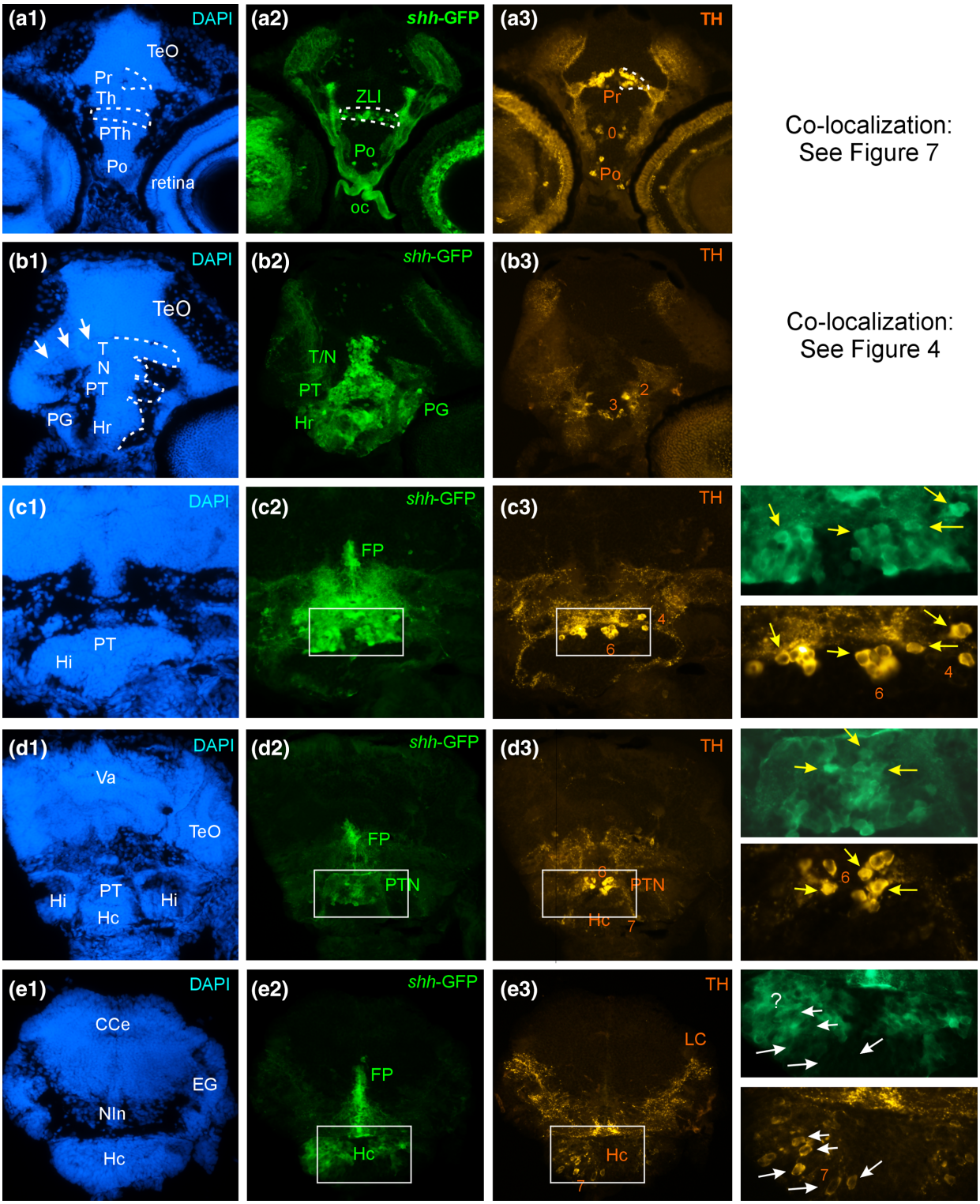


FIGURE 8 Sagittal analysis: Focus on diencephalon of adult transgenic *shh*-GFP zebrafish. Four parasagittal sections of a *shh*-GFP transgenic zebrafish brain through preoptic region, prethalamus/thalamus, pretectum, posterior tuberculum and hypothalamus showing nuclear DAPI stain (a1–d1), *shh*-GFP (a2–d2) and tyrosine hydroxylase (TH; a3–d3) immunostains. Note the absence of overlap of *shh*-GFP and TH in preoptic region, pretectum, prethalamus, and caudal/ventral zone of periventricular hypothalamus whereas in TPp-m cells these two markers colocalize (yellow arrows). For colocalization in adult PVO and TPp-p, see Figure 4 and text. Abbreviations: ac, anterior commissure; CM, corpus mamillare; EF, endfeet; Ha, habenula; Hc/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; LR, lateral recess (hypothalamus); mfb, medial forebrain bundle; Nmlf, nucleus of the medial longitudinal fascicle; oc, optic chiasma; pc, posterior commissure; poc, postoptic commissure; PPa/PPp, anterior/posterior parvocellular preoptic nucleus; PPr, periventricular pretectum; PR, posterior recess (hypothalamus); PT, posterior tuberculum; PTh, prethalamus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; SC, suprachiasmatic nucleus; T, midbrain tegmentum; TeO, optic tectum; TeV, tectal ventricle; Th, thalamus; TPp-p, parvocellular cell part of periventricular posterior tubercular nucleus; TPp-m, magnocellular (pear-shaped) cell part of periventricular posterior tubercular nucleus; ZLI, zona limitans intrathalamica [Color figure can be viewed at wileyonlinelibrary.com]

1993; Ghanem et al., 2007; Marín & Rubenstein, 2001; Marín, Anderson, & Rubenstein, 2000; Qiu, Shimamura, Sussel, Chen, & Rubenstein, 1998; Sussel, Marín, Kimura, & Rubenstein, 1999). The *Nkx2.2* expression does not follow this telencephalic *shh* domain dorsally. However, the telencephalic *Nkx2.1* expression domain overlaps with that of *Shh* and extends even further dorsally throughout the entire MGE (Figure 10c; Puelles et al., 2000; Shimamura et al., 1995).

The *Nkx6.1* gene codes for another longitudinally expressed homeobox transcription factor dorsal to the *Shh* domain (Figure 10c; Qiu et al., 1998). From spinal cord, up to hindbrain the *Nkx6.1* stripe overlaps with that of *Nkx2.2*, but extends more dorsally than the latter. Many motoneurons coexpress *Nkx6.1* and *Islet1*. In the midbrain and in P1/P2, *Nkx6.1* overlaps with *Shh* expression, but *Nkx2.2* expression is now dorsal to it. A thin double-positive *Nkx6.1* and *Nkx2.2* stripe extends from P3 into the hypothalamus, here dorsal to both *shh* and *Nxk2.1* domains (Qiu et al., 1998). Expression of *Nkx6.2* is similar to *Nkx6.1* in hindbrain and midbrain, but absent in spinal cord



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and in P3 through hypothalamus (Qiu et al., 1998). However, *Nkx6.2* is again expressed in the most dorsal MGE (Fogarty et al., 2007).

The subpallial telencephalon includes the MGE (the future pallidum) and dorsal to it the lateral ganglionic eminence (LGE, the future striatum) and both are molecularly characterized by expression of homeodomain-containing genes such as various *Dlx* paralogs (i.e., *Dlx1/2* and *Dlx5/6*; Panganiban & Rubenstein, 2002), *Gsh2* and *Islet1* genes, as well as the basic-Helix–Loop–Helix (bHLH) gene *Ascl1* (mouse: *Mash1)*, followed more dorsally by expression of pallial genes, such as *Pax6*, *Emx*, or *Tbr* genes, as well as the bHLH genes *Ngn1/2* and *Nrd* (Figure 10B1,B2; Casarosa, Fode, & Guillemot, 1999; Corbin, Gaiano, Machold, Langston, & Fishell, 2000; Englund et al., 2005; Fode et al., 2000; Horton, Meredith, Richardson, & Johnson, 1999; Ma, Sommer, Cserjesi, & Anderson, 1997; Muzio et al., 2002; Osório, Mueller, Rétaux, Vernier, & Wullimann, 2010; Parras et al., 2002, 2004; Price et al., 1992; Puelles et al., 2000; Schuurmans & Guillemot, 2002; Sommer, Ma, & Anderson, 1996; Stoykova, Treichel, Hallonet, & Gruss, 2000; Toresson & Campbell, 2001; Toresson, Potter, & Campbell, 2000; Torii et al., 1999; Yun, Garel, Fischman, & Rubenstein, 2003). *Islet 1* expressing *Dlx2* positive cells are dynamically emerging over time both in MGE and LGE (Toresson et al., 2000; Wang & Liu, 2001; Yu, Fotaki, Mason, & Price, 2009) with those of LGE developing into GABAergic striatal projection neurons and those of MGE into cholinergic striatal interneurons (Flames et al., 2007; Marín et al., 2000; Olsson, Björklund, & Campbell, 1998; Pilz et al., 2013; Stenman, Toresson, & Campbell, 2003).

Dlx 1/2 and *Dlx5/6* genes have indispensable overlapping and sequential roles in the differentiation of GABAergic cells in all subpallial divisions (i.e., striatal LGE, pallidal MGE, and CGE, the caudal ganglionic eminence, the future subpallial amygdala; Panganiban & Rubenstein, 2002; Wonders & Anderson, 2006). However, some genes, such as *Nkx2.1* (see above), *Gsh1* (Corbin et al., 2000; Toresson & Campbell, 2001; Yun et al., 2003) and the LIM/homeodomain genes *Lhx6* and *Lhx7* as well as the homeobox gene *Gbx1* are exclusively expressed in MGE and septum, but not in LGE (Asbreuk et al., 2002; Grigoriou, Tucker, Sharpe, & Pachnis, 1998; Marín et al., 2000; Stoykova et al., 2000). *Nkx2.1* and *Lhx6* are essential for tangential migration of MGE cells into LGE or cortex (Alifragis, Liapi, & Parnavelas, 2004; Marín et al., 2000; Wonders & Anderson, 2006), whereas *Lhx7* (synonymous to *Lhx8*; Zhao et al., 2003) has an additional role in conferring the transmitter phenotype to cholinergic-GABAergic striatal and basal forebrain neurons (Asbreuk et al., 2002; Fragkouli et al., 2005; Fragkouli, Pachnis, & Stylianopoulou, 2006; Manabe et al., 2005; Marín et al., 2000; Mori et al., 2004; Wonders & Anderson, 2006). *Dlx1/2* genes act upstream of these MGE expressed genes, because—when mutant—the generation of all cortical GABAergic interneurons is suppressed (Anderson, Eisenstat, Shi, & Rubenstein, 1997; Nery, Corbin, & Fishell, 2003). While the MGE is the predominant provider of tangentially migrating interneurons into striatum (cholinergic) and cortex (GABAergic), the LGE and CGE also partake later in this tangential migration process. This follows from *Nx2.1* mutants where most cortical interneurons are gone, but some persist (Anderson, Marín, Horn, Jennings, & Rubenstein, 2001; Corbin, Nery, & Fishell, 2001; Nery et al., 2003; Nery, Fishell, & Corbin, 2002; Tamamaki, Fujimori, & Takauji, 1997; Wonders & Anderson, 2006). Regarding these cortical interneurons, the MGE and CGE (*Lhx6* cells) provide somatostatin, parvalbumin or calbindin positive inhibitory interneurons which are physiologically different, whereas the dLGE (which expresses *Nkx6.2*) produces double calretinin/somatostatin positive ones (Butt et al., 2005; Fogarty et al., 2007; Nery et al., 2002; Wonders & Anderson, 2006). The CGE is heterogenous and also provides calretinin cells, originating from its *Nkx2.1/Lhx6* negative domain as these interneurons remain present in *Nkx2.1* mutants (Nery et al., 2002; Xu, Cobos, De La Cruz, Rubenstein, & Anderson, 2004). The POA and AEP share much of pallidal-type gene expression (*Ascl1*, *Gsh1/2*, *Dlx1/2/5/6*, *Lhx6/7*) (Asbreuk et al., 2002).

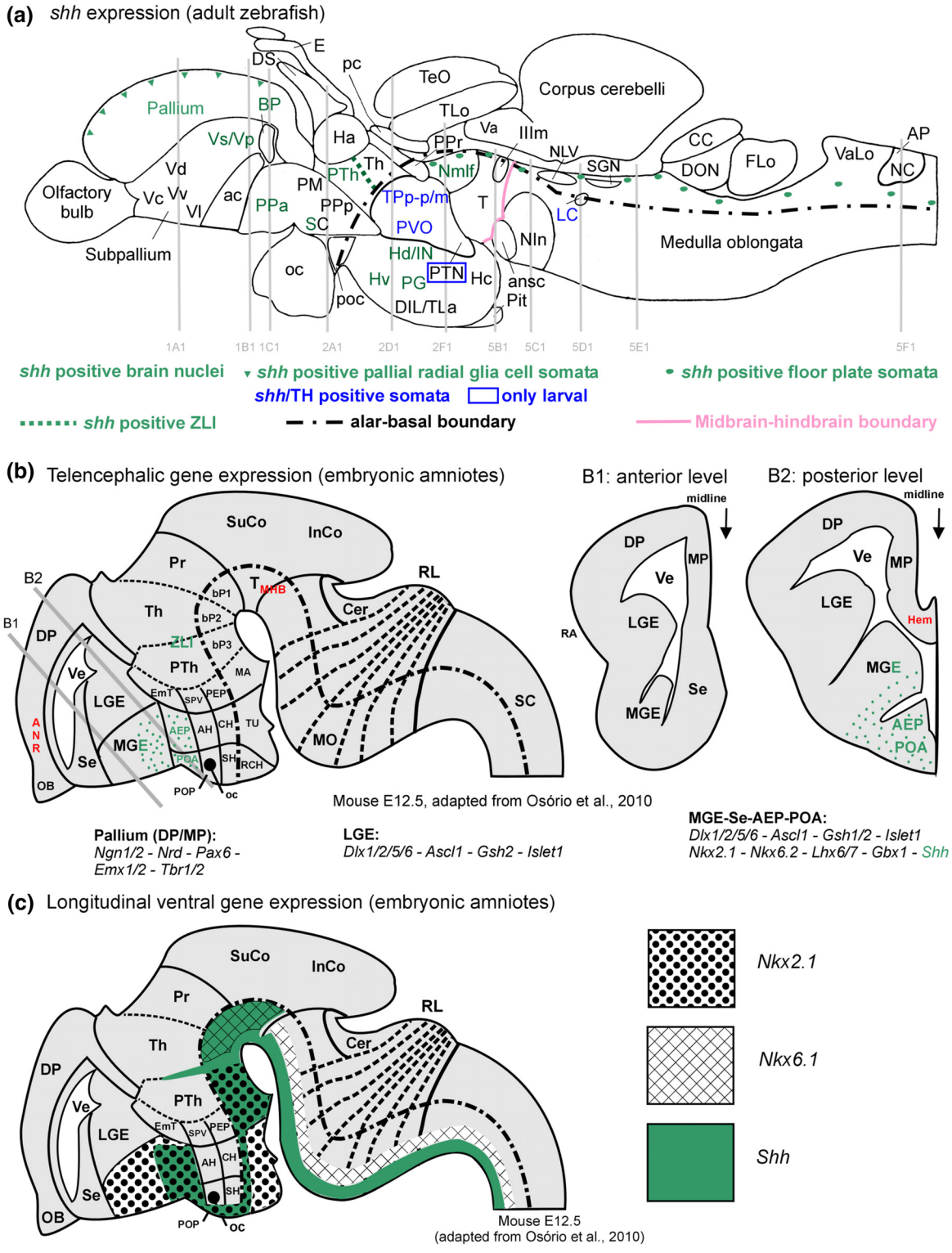
Two conclusions follow from these studies. First, the differential ventrodorsal gene expression patterns along the neuraxis share similarities into the telencephalon (e.g., *Shh*; *nkx genes*) suggesting ventrodorsal induction also there. Second, local differences in longitudinal gene expression (e.g., *Nkx2.1* vs. *Nkx6.1*) suggest that the mechanisms of induction of ventral phenotypes along the anteroposterior axis differ (Balaskas et al., 2012; Litingtung & Chiang, 2000; Placzek & Briscoe, 2005). We will focus on data directly relevant to forebrain ventralization, in particular, the telencephalon, for which we report new results in the zebrafish.

4.3.3 | What is the role of SHH in telencephalic gene expression induction and repression?

The differential transcription factor expression described above for amniotes presents a distinctly nested anteroposterior and ventrodorsal

FIGURE 9 Expression of *shh*-GFP and tyrosine hydroxylase in the late larval/juvenile (13d) zebrafish brain. Transverse sections run from alar diencephalon (a; pretectum/thalamus/prethalamus), through posterior tuberculum (b), into three levels of hypothalamus (c–e). Designations and Arabic numbers are used as established for larval zebrafish brain by Rink and Wullimann (2002). White arrows in (b1) point to tectal ventricle. Note that unlike in the adult zebrafish brain, the posterior tuberal nucleus (PTN) contains *shh*-GFP cells, which colocalize with tyrosine hydroxylase (TH) and, maybe also the caudal zone of the periventricular hypothalamus (Hc, around posterior recess). In magnifications shown in most right column, single cells double-labeled for TH and *shh*-GFP are indicated by yellow arrows and labeled only for TH by white arrows. See text for details. Abbreviations: CCe, corpus cerebelli; EG, eminentia granularis; FP, floor plate; Hr/Hi/Hc, rostral/intermediate/caudal hypothalamus; LC, locus coeruleus; N, area of the nucleus of the medial longitudinal fascicle; NIn, interpeduncular nucleus; oc, optic chiasma; PG, preglomerular complex; Po, preoptic region; Pr, pretectum; PT, periventricular posterior tuberculum; PTh, prethalamus (0); PTN, posterior tuberal nucleus (6); T, midbrain tegmentum; TeO, optic tectum; Th, (dorsal) thalamus; Va, valvula cerebelli; ZLI, zona limitans intrathalamica; 0, prethalamic dopamine cells (zona incerta); 2, 4, anterior and posterior magnocellular (pear-shaped) dopamine cells of periventricular posterior tuberculum; 3, paraventricular organ dopamine cells; 6, posterior tuberal nucleus dopamine cells; 7, caudal hypothalamic dopamine cells [Color figure can be viewed at wileyonlinelibrary.com]

forebrain patterning. These patterns principally result from the combined spatiotemporally dynamic activity of inductive signals (morphogens) from various sources (signaling centers), followed by cross-regulatory interactions of homeodomain and bHLH gene activity (Campbell, 2003; Marín & Rubenstein, 2001; Schuurmans & Guillemot, 2002). The signaling centers include in addition to the ventral notochord/prechordal mesoderm and later ventral neural tube (SHH), also the anterior neural ridge (fibroblast growth factor, FGF8), the dorsal neural tube midline/cortical hem (bone morphogenetic protein 4-BMP4 and Wnt3a), the lateral mesoderm (retinoic acid, RA) and the transverse zona limitans intrathalamica (SHH) as



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well as more caudally the midbrain–hindbrain boundary (FGF8). The dorsal BMP and Wnt signals have a dorsalizing effect (Lupo et al., 2006; Storm et al., 2006; but see BMP7 below) counteracted by ventralizing SHH effect (see below). This activity results in ventral forebrain neurons and, more caudally, in motor neurons both apparent by *Islet1* expressing cells laterally adjacent to the longitudinal *Shh* domain (Ericson, Muhr, Jessel, et al., 1995; Ericson, Muhr, Placzek, et al., 1995). Except for the telencephalon, *Shh* expression precedes that of *Islet1* (Echelard et al., 1993; Ericson, Muhr, Placzek, et al., 1995). Neural plate explants from different anteroposterior levels exposed to a SHH source are induced to coexpress different gene markers with *Islet1* (*Nkx2.1* only in forebrain, including telencephalon; *LIM1* in basal diencephalon; *SC1* and *Nkx6.1* in midbrain, hindbrain, spinal cord; Roelink et al., 1994; Ericson, Muhr, Placzek, et al., 1995; Qiu et al., 1998). Additionally, non-*Islet1* positive populations (such as serotonin and dopamine cells; Yamada, Placzek, Tanaka, Dodd, & Jessell, 1991; Hynes, Poulsen, Tessier-Lavigne, & Rosenthal, 1995) are induced by SHH in midbrain/hindbrain explants. Also, *Nkx2.2* is induced in neural tube explants by SHH (Qiu et al., 1998), but not *Nkx6.1* which is induced by additional signals from the notochord (Qiu et al., 1998).

Although various studies show that *Nkx2.1* expression is induced in forebrain neural plate explants exposed to SHH (see above), the pallidal *shh* domain is downstream of *Nkx2.1* (Qiu et al., 1998; Shimamura & Rubenstein, 1997). This follows from studies on *Nkx2.1* mutants, where *Shh* expression is absent in MGE and hypothalamus (except for POA), but present from midbrain to spinal cord (Sussel et al., 1999). Thus, initial pallidal *Nkx2.1* expression is induced by earlier SHH influence from prechordal mesoderm (Dale et al., 1997; Shimamura & Rubenstein, 1997) together with FGF8 from the anterior neural ridge (Storm et al., 2006; Wonders & Anderson, 2006). Furthermore, in the forebrain, high concentrations of SHH induce BMP7 (from prechordal mesoderm) which in turn supresses *Nkx6.1* and promotes *Nkx2.1* expression (Anderson, Lawrence, Stottmann, Bachiller, & Klingensmith, 2002; Dale et al., 1997; Pera & Kessel, 1997; Qiu et al., 1998). Also, different types of tangentially migrating MGE neurons are dependent on differential SHH levels (Xu et al., 2010).

Beyond the early SHH influence on ventralization of the telencephalon through *Nkx2.1* (i.e., inducing MGE features), later various *Dlx* and *Islet1/2* gene expression is also induced in LGE (Kohtz, Baker, Corte, & Fishell, 1998). For example, SHH induces different LGE cell populations to express singly or combined *Dlx* and *Mash1* as well as *Dlx* and *Islet1/2*, with *Islet1* always only in postmitotic cells (Kohtz et al., 2001). In contrast, the expression of certain dorsal (pallial) genes is prohibited by SHH in the subpallium. For example, *Gsh2* expression in LGE/MGE is SHH dependent and has a particularly strong role for correct striatal development. In *Gsh2* (but not in MGE-specific *Gsh1*) mutants, typical striatal gene expression is absent (*Mash/Dlx/Islet1*) and pallial genes (*Pax6*, *Ngn1/2*) expand ventrally into striatum (Corbin et al., 2000; Toresson & Campbell, 2001; Toresson, Potter, & Campbell, 2000; Yun et al., 2003). Furthermore, in *Shh* mutants, pallial *Emx1* gene expression expands into the ventral (striatal) area (Chiang et al., 1996).

In addition to this information in amniotes, a similar situation is present in basal anamniote sarcopterygians (frogs, salamanders, and

FIGURE 10 Summary schemata show (a) sagittal view of adult zebrafish brain with *shh*-GFP positive structures indicated in color. See figure for color code of zebrafish *shh*-GFP structures either singly labeled (green) or additionally double-labeled for tyrosine hydroxylase (TH; blue). Blue square around PTN indicates that double-labeled TH cells are present in larvae only. (b) telencephalic gene expression in embryonic amniotes. (b) Schematic sagittal section of a E12.5 mouse brain. (B1) corresponding anterior telencephalic transverse section. (B2) corresponding posterior telencephalic transverse section. Red letters designate signaling centers issuing FGF8 (ANR, MHB) or MPB4 (Hem), green letters (and green dots) indicate signaling centers issuing SHH. Additionally, retinoic acid (RA) acts from lateral mesoderm into the telencephalon (Lupo, Harris, & Lewis, 2006). Stippled lines in (b) indicate prosomeric or rhombomeric boundaries and semicolon line indicates anteroposterior CNS axis along alar-basal boundary. Gray lines in (a) and (b) indicate levels of transverse sections shown in various figures. See text for details and citations for gene expression. (c) Longitudinal ventral gene expression in embryonic amniotes. Schematic sagittal section of a E12.5 mouse brain on which three major gene expression domains are summarized, *Shh* (green), *Nkx2.1* (black dots), *Nkx6.1* (cross-hatched; after Qiu, Shimamura, Sussel, Chen, & Rubenstein, 1998; see text for details). Abbreviations: ac, anterior commissure; AEP, anterior entopeduncular area (SHH); AH, anterior hypothalamus; ANR, anterior neural ridge (FGF8); ansc, ansular commissure; AP, area postrema; BP, basal pallidum; bP1, basal plate of prosomere 1; bP2, basal plate of prosomere 2; bP3, basal plate of prosomere 3; Cer, cerebellum; CC, crista cerebellaris; DIL, diffuse nucleus of inferior lobe; DON, descending octaval nucleus; DP, dorsal pallium (Isocortex); DS, saccus dorsalis; EmT, eminentia thalami; FLo, facial (sensory) lobe; FP, floor plate; Ha, habenula; Hc/Hd/Hv, caudal/dorsal/ventral zone of periventricular hypothalamus; IN, intermediate hypothalamic nucleus; InCo, inferior colliculus; LC, locus coeruleus; LGE, lateral ganglionic eminence; MA, mammillary hypothalamus; MGE, medial ganglionic eminence; MHB, midbrain–hindbrain boundary (FGF8); MO, medulla oblongata (rhombencephalon without cerebellum); MP, medial pallium (hippocampus); NC, commissural nucleus of Cajal; NIn, nucleus interpeduncularis; NLV, nucleus lateralis valvulae; Nmlf, nucleus of the medial longitudinal fascicle; OB, olfactory bulb; oc, optic chiasma; pc, posterior commissure; PEP, posterior entopeduncular area; PG, preglomerular complex; Pit, pituitary; PM, magnocellular preoptic nucleus; POA, anterior preoptic area (SHH); POP, posterior preoptic area; poc, postoptic commissure; PPa/PPp, anterior/posterior parvocellular preoptic nucleus; Pr, pretectum; PPr, periventricular pretectum; PTh, prethalamus (formerly ventral thalamus); PTN, posterior tuberal nucleus; PVO, paraventricular organ; RA, retinoic acid; RCH, retrochiasmatic hypothalamus; RL, rhombic lip; SC, suprachiasmatic nucleus/spinal cord in b; Se, septum; SGN, secondary gustatory nucleus; SH, suprachiasmatic area; SPV, supraoptic/paraventricular area; SuCo, superior colliculus; T, midbrain tegmentum; TeO, optic tectum; Th, thalamus (formerly dorsal thalamus); TLa, torus lateralis; TLo, torus longitudinalis; TPp-p/m, parvocellular/magnocellular cell part of periventricular posterior tubercular nucleus; TU, tuberal hypothalamus; Va, valvula cerebelli; VaLo, vagal (sensory) lobe; Vc/Vd/Vl/Vp/Vs/Vv, central/dorsal/lateral/ postcommissural/supracommissural/ ventral nucleus of ventral telencephalon; Ve, telencephalic brain ventricle; ZLI, zona limitans intrathalamica (SHH); IIIm, oculomotor nerve nucleus [Color figure can be viewed at wileyonlinelibrary.com]

lungfishes) regarding forebrain expression patterns in general (Domínguez, González, & Moreno, 2014; Domínguez, Morona, González, & Moreno, 2013; González, Morona, Moreno, Bandín, & López, 2014; Medina, Brox, Legaz, García-López, & Puelles, 2005; Moreno & González, 2011), as well as for *Shh* (exhibiting a small basal pallidal domain; Domínguez, González, & Moreno, 2010), *Nkx2.1* (pallidum; González, López, & Marín, 2002; González, López, Sánchez-Camacho, & Marín, 2002; Van den Akker, Brox, Puelles, Durston, & Medina, 2008; Moreno et al., 2018), *Lhx7* (pallidum; Moreno, Bachy, Rétaux, & González, 2004)) and *Islet1* (striatum/pallidum; Moreno, Domínguez, Rétaux, & González, 2008; Moreno et al., 2018), as well as regarding the process of tangential migration (Moreno, González, & Rétaux, 2008).

The basic message from these studies is that SHH (from mesodermal and ventral neural tube sources) has a pivotal role in ventralization of the tetrapod/sarcopterygian forebrain including the telencephalon. As mentioned above, an early pallidal *shh* domain is elusive in zebrafish. However, in cavefish, a small pallidal *shh* domain has been documented and confirmed by expression of characteristic pallidal genes *lhx6/7* (Menuet, Alunni, Joly, Jefferey, & Rétaux, 2007). Our new finding of a zebrafish pallidal *shh*-GFP expression domain shows that this domain is also present in zebrafish. Its late developmental emergence, however, has prohibited a functional investigation (see section 1).

In teleosts, general forebrain expression patterns also agree with those in tetrapods, for example regarding pallial versus subpallial gene expression (reviewed in Wullimann, 2009; Mueller & Wullimann, 2009, 2016), as well as regarding the process of tangential migration (Mueller et al., 2008; Mueller, Vernier, & Wullimann, 2006). Two paralogs of *nkx2.1* (*a/b*) exist in zebrafish (Manoli & Driever, 2014; Rohr, Barth, Varga, & Wilson, 2001), with *nkx2.1b* expressed in the embryonic/larval pallidum and *nkx2.1a* in hypothalamus. This is confirmed by larval expression of both "pallidal" genes *lhx6/lhx7* which are expressed in a ventral subdivision of the dorsal nucleus of the zebrafish ventral telencephalon (Mueller et al., 2008) and by corresponding adult pallidal *islet1* expression (Vdv; Baeuml et al., 2019). As discussed in this previous paper, we interpret the adult *islet1* expression extending into Vdv as defining the zebrafish pallidum, as also applies to basal telencephalic *nkx2.1b* domain, both of which are erroneously assigned to the ventral nucleus of the ventral telencephalon by Ganz et al. (2012).

4.3.4 | Pallial *shh*-GFP radial glia cells in medial zone of dorsal telencephalon (Dm)

In addition to the long-known basal longitudinal expression of *shh* in the mesodermal notochord/prechordal plate and floor plate/ventral forebrain (see above), evidence later arose in amniotes for previously unknown early *shh* expression in dorsal (i.e., alar) CNS regions (Dahmane et al., 2001; Dahmane & Ruiz i Altaba, 1999; Kriegstein & Alvarez-Buylla, 2009; Ruiz i Altaba, Palma, & Dahmane, 2002) such as the mammalian isocortex, the superior colliculus, and the cerebellum. In the cerebellum, *shh* expressing Purkinje cells act in transit amplification in the external granular layer. In the early mammalian isocortex, *shh* expression was reported in radial glia cells (Wang, Hou, & Han, 2016) and other cortical cells in intermediate zone, subplate, and deep cortical plate cells (Radonjic et al., 2016). Also, *shh* is more strongly expressed in gyrencephalic species (primates) than lissencephalic mammalian brains (rodents) in the developing cortical ventricular zone and apparently plays a role in the multiplication of progenitors (outer radial glia/intermediate progenitors; Han, 2016).

We demonstrated recently that *shh* expressing cells are also found in the larval zebrafish optic tectum and cerebellum (Biechl et al., 2016), but no such cells are seen in the larval pallial telencephalon. However, here we show newly a *shh*-GFP expressing population in the adult zebrafish pallial telencephalon, that is, pallial radial glia cells. Various studies have shown that mitotic stem cells (radial glia) exist in the adult zebrafish telencephalon along the subpallial and pallial ventricular lining (e.g., Chapouton, Jagasia, & Bally-Cuif, 2007; Diotel et al., 2015; Kaslin, Ganz, & Brand, 2007; Lillesaar, Stigloher, Tannhäuser, Wullimann, & Bally-Cuif, 2009; Lindsey, Darabie, & Tropepe, 2012; März, Schmidt, Rastegar, & Strähle, 2010; Than-Trong & Bally-Cuif, 2015). However, to the best of our knowledge, a role for *shh* has not been shown in adult telencephalic stem cells. Recently, zebrafish telencephalic stem cells were investigated with a transcriptomic approach (Cosacak et al., 2019) and shown to be organized into molecularly separable populations that are clearly closely correlated with earlier established neuroanatomical divisions (Wullimann et al., 1996). One of these stem cell populations is in the medial zone of the pallial telencephalon (Dm; considered the pallial amygdala; Portavella et al., 2002, 2004; Wullimann & Mueller, 2004; Lal et al., 2018) which is characterized by marker genes *pou3f1* and *dmrta2* (Cosacak et al., 2019). It appears that we show here with *shh*-GFP specifically this population of molecularly defined radial glia cells within the medial zone of the dorsal (pallial) telencephalon (Dm; Figure 1). Thus, while there is no support for an early role for *shh* in the developing zebrafish pallium in the literature, a later role for *shh* in the adult pallium is suggested by our finding of *shh*-GFP positive radial glia cells in Dm and their function will be interesting to be studied in the future in the context of the known continuing proliferative activity in the zebrafish pallium (see citations above).

4.4 | Analysis of *shh*-GFP in comparison to *islet1*-GFP suggests that most forebrain *shh* cells remain at the ventricle and are not integrated into parenchymal tissue

The analysis of transverse zebrafish brain sections reveals that *shh*-GFP positive cells are generally located close to the ventricular lining. This is evident for the floor plate cells of spinal cord and hindbrain which remain the only *shh*-GFP cells there also in the adult brain with the exception of a few locus coeruleus cells. Floor plate cells are also seen in the adult midbrain tegmentum (T; Figures 6 and 7), although in larvae many more cells appear to be present there compared to the

hindbrain (Figure 9; Biechl et al., 2016; Baeuml et al., 2019). A position close to the ventricle is also seen for most *shh*-GFP cells in the forebrain, starting with the most caudal ones in the basal plate of P1 which partly are clearly identifiable as floor plate cells (see above). However, there are a number of more migrated *shh*-GFP cells seen in this area of the nucleus of the medial longitudinal fascicle (Nmlf). Such peripherally migrated *shh*-GFP positive cells become more abundant at the level of the ZLI and anterior to it in the area of the posterior tuberculum (Figure 2). Because dopamine cells in this area form various well-known brain nuclei, we consider sections additionally stained for tyrosine hydroxylase (TH) in detail in section 4.2. However, in the hypothalamus, *shh*-GFP cells remain again rather close to the ventricle and this is also true for the telencephalon (see above).

We have recently analyzed in detail the expression of *islet1* using a transgenic *islet1*-GFP line (Baeuml et al., 2019) and since *islet1* expressing cells are generally considered to be influenced by ventricularly located SHH secreting cells (see above), we looked at comparable levels of *islet1*-GFP and *shh*-GFP transverse zebrafish brain sections at 3 months and evaluated qualitatively their positions with respect to the ventricle. Clearly, at telencephalic and preoptic into hypothalamic levels, *islet1*-GFP cells are always located more peripherally remote from the ventricle than *shh*-GFP cells (see Figure 3, where dashed lines enclose more ventricularly located *shh*-GFP cells in Figure 3g through Figure 3l and exclude more migrated *islet*-GFP cells in Figure 3a through Figure 3f). Thus, the zebrafish diencephalon is largely similar compared to the more posterior brain with respect to the ventricular position of *shh*-GFP cells which do not migrate into the brain parenchyma. This is in line with the working hypothesis that *shh* cells act through this morphogen on nearby cells to express *islet1*.

Also in the (alar) prethalamus, the most ventricularly located layer 1 is free of *islet1*-GFP, whereas many *shh*-GFP cells are present there. In contrast, more peripheral prethalamic layers 2 and 3 contain both GFP gene markers. However, because TH is colocalized only with *islet1*-GFP (Baeuml et al., 2019), but never with *shh*-GFP (this study), these are not the same cells. Thus, generally *shh*-GFP and *islet1*-GFP label in adult zebrafish brain structures do not colocalize on the cellular level (for the only possible exception see section 3.2 and Table 2).

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Mario F. Wullimann https://orcid.org/0000-0001-9292-2851

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